

for particle-mediated (PowderJect) DNA vaccines without any detectable local or systemic toxic effects. Results to date are derived from experiments with DNA vaccine vectors from influenza virus, hepatitis B virus, HSV-2 and HIV-1 using mice and domestic pigs. Typical effects observed in mice following use of CT-encoding adjuvant vectors include a 10-100-fold reduction in the antigen-specific IgG1-to-IgG2a ratio and a 10-fold enhancement in CD8+ IFN-gamma ELISPOT responses. Following use of LT-encoding vectors, an even stronger Th1 effect was observed with a greater enhancement of T cell responses and a further reduction in the IgG1-to-IgG2a ratio resulting in a marked abundance of IgG2a versus IgG1 antibodies. In mice, the effect of CT and LT vector adjuvants on total IgG antibody levels was antigen dependent, but the IgG1-to-IgG2a ratio was always sharply reduced in adjuvant groups. In early studies in pigs, data are limited to antibody titers but a marked augmentation of influenza virus antigen-specific responses was observed. The use of particle-mediated DNA vaccine delivery to the skin allows for the realization of the full adjuvant potential of CT and LT adjuvants without any apparent local or systemic toxicity.

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 AN 1998031625 EMBASE
 TI Gene therapy for infectious diseases.
 AU Bunnell B.A.; Morgan R.A.
 CS R.A. Morgan, Clinical Gene Therapy Branch, National Human Genome Research Inst., Building 10, 9000 Rockville Pike, Bethesda, MD 20892-1851, United States. rmorgan@nhgri.nih.gov
 SO Clinical Microbiology Reviews, (1998) Vol. 11, No. 1, pp. 42-56. .
 Refs: 165
 ISSN: 0893-8512 CODEN: CMIREX
 CY United States
 DT Journal; General Review
 FS 004 Microbiology
 026 Immunology, Serology and Transplantation
 LA English
 SL English
 ED Entered STN: 12 Feb 1998
 Last Updated on STN: 12 Feb 1998
 AB Gene therapy is being investigated as an alternative treatment for a wide range of infectious diseases that are not amenable to standard clinical management. Approaches to gene therapy for infectious diseases can be divided into three broad categories: (i) gene therapies based on nucleic acid moieties, including antisense DNA or RNA, RNA decoys, and catalytic RNA moieties (ribozymes); (ii) protein approaches such as transdominant negative proteins and single-chain antibodies; and (iii) immunotherapeutic approaches involving genetic vaccines or pathogen-specific lymphocytes. It is further possible that combinations of the aforementioned approaches will be used simultaneously to inhibit multiple stages of the life cycle of the infectious agent.

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 L3 1289 S L2 AND "LIFE CYCLE"
 L4 15 S L1 AND L2
 E ROIZMAN BERNARD/AU
 L5 713 S E2, E3

L6 677 S L5 AND L2
 L7 631 S L2 AND ("NUCLEIC ACID" OR "POLYNYCLEOTICE") AND VACCINE
 L8 3 S L3 AND L7
 L9 57 S L6 AND (VACCINE OR VECTOR)
 L10 3 S L9 AND ICP
 L11 4534 S (ICP0 OR ICP4 OR ICP22 OR ICP27)
 L12 4431 S L2 AND L11
 L13 73 S L3 AND L11
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Replicative Oncolytic Herpes Simplex Viruses in Combination Cancer Therapies

Dawn E. Post^{1,*}, Giulia Fulci², E. Antonio Chiocca², and Erwin G. Van Meir^{1,*}

¹Laboratory of Molecular Neuro-Oncology, Departments of Neurosurgery, Hematology/Oncology and Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA, 30322, USA, ²Molecular Neuro-Oncology Laboratories, Massachusetts General Hospital-East Building, CNY6, 13th Street, Charlestown, MA, 02129, USA

Abstract: Viruses that kill the host cell during their replication cycle have attracted much interest for the specific killing of tumor cells and this oncolytic virotherapy is being evaluated in clinical trials. The rationale for using replicative oncolytic viruses is that viral replication in infected tumor cells will permit in situ viral multiplication and spread of viral infection throughout the tumor mass thus overcoming the delivery problems of gene therapy. Improved understanding of the life cycle of viruses has evidenced multiple interactions between viral and cellular gene products, which have evolved to maximize the ability of viruses to infect and multiply within cells. Differences in viral-cell interactions between normal and tumor cells have emerged that have led to the design of a number of genetically engineered viral vectors that selectively kill tumor cells while sparing normal cells. These viruses have undergone further modifications to carry adjunct therapy genes to increase their anti-cancer abilities. Since these viruses kill cells by oncolytic mechanisms differing from standard anticancer therapies, there is an opportunity that synergistic interactions with other therapies might be found with the use of combination therapy. In this review, we focus on the oncolytic Herpes Simplex Virus-1 (HSV-1) vectors that have been examined in preclinical and clinical cancer models and their use in combination with chemo-, radio-, and gene therapies.

Keywords: Gene therapy, tumors, oncolytic virus, herpes simplex virus 1, radiation, chemotherapy

INTRODUCTION

Immense progress has been made in unraveling the genetic defects and altered molecular mechanisms contributing to cancer formation. Cancer can be detected today at earlier stages following technical advances in detection modalities such as imaging and early marker detection. In spite of such immense progress cancer remains a devastating disease worldwide. Treatment modalities have improved with development of novel chemotherapeutic regimens and optimized radiotherapies. Cancer patients diagnosed with primary tumors such as malignant gliomas, pancreatic and hepatic cancers as well as those showing malignant or metastatic cancer still have poor a very prognosis. There is an urgent need for the development of novel anti-cancer approaches. Undoubtedly, these treatments will need to be used in combination to be most effective against such a complex disease.

One novel therapeutic modality that holds much promise is the use of oncolytic viruses that can infect and undergo selective replication in cancer cells. These viruses can be roughly divided in two groups [Fulci and Chiocca, 2002]: (1) wild-type viral strains that have intrinsic cancer selective activation (Newcastle disease virus, vesicular stomatitis virus, vaccinia virus and autonomous parvovirus) and (2) viral-engineered variants that have acquired selective

antitumor effects (Adenovirus, HSV, and poliovirus). Preclinical research has demonstrated that these viruses have antineoplastic activity and the evaluation of their safety, effectiveness and potential limitations is currently being examined in clinical trials. The perfect oncolytic virus would have i) the ability to specifically kill tumor cells while sparing normal cells, ii) show effective lysis and propagation within a tumor, iii) carry low or bearable toxicity, iv) be easy to engineer, v) and produce in large quantities, vi) genomically stable, thus avoiding the generation of toxic, undesirable mutants that could pose a danger, vii) incorporate a "fail-safe" mechanism for inactivation, and viii) absence of potential spread to the general population. Several of the current oncolytic herpes viruses fulfill many of these criteria and further modifications of their genomes to improve them even further are currently ongoing.

During the replicative life cycle of HSV its gene products are involved in multiple interactions with cellular proteins, which optimize the ability of the virus to multiply within cells. Understanding such interactions has permitted the modification of HSVs by genetic engineering to selectively reduce their ability to replicate in normal, but not tumor cells. These modifications exploit differences between normal and tumor cells such as the expression of viral receptors or the differential activation of transcription factors. These attenuated viruses harbor further modifications so they also serve as therapeutic gene delivery vehicles with augmented anti-cancer efficacy. A number of these engineered HSV strains have been used in multimodal combination therapies including gene, radio-, and chemo-

*Address correspondence to these authors at the Laboratory of Molecular Neuro-Oncology, Departments of Neurosurgery, Hematology/Oncology and Winship Cancer Institute, Emory University School of Medicine, Atlanta, GA, 30322, USA; Tel: 404-778-5227; Fax: 404-778-5240; E-mail: dephelp@emory.edu or evanmeir@emory.edu

therapies and have shown augmented therapeutic effects compared to single treatments. Here, we review the oncolytic HSV-1 vectors that have undergone preclinical and clinical testing and the use of these viruses in combination therapies for cancer.

1. HSV-1

The HSV-1 genome is composed of a linear double stranded DNA molecule of approximately 152 Kb. Following infection, the viral DNA circularizes in the nucleus and a tightly regulated temporal program of HSV gene transcription is initiated [Roizman and Sears, 1996]. Viral genome transcription starts with five immediate early genes that encode factors responsible for the transcription of the remaining viral genes and for evasion of the host immune system. Two of these genes, ICP4 and ICP27, are essential for the initiation of viral gene expression and null mutants do not undergo viral replication. Early gene expression initiates the onset of viral DNA synthesis and many early viral proteins are responsible for DNA metabolism while the late genes encode virion structural proteins.

To date oncolytic HSVs all derive from HSV-1. Wild type HSV-1 is responsible for mucocutaneous lesions of the mouth, face and eyes [Whitley, 1996]. Following an initial local infection, HSV-1 can enter the sensory neurons innervating the cells of mucosal membranes where it remains in a latent phase, in which the virus genome is transcriptionally silent, except for one gene (the *LAT* gene), whose function remains unknown. Environmental stress factors can induce re-activation of HSV-1, which ultimately triggers the recurrence of oral mucocutaneous lesions. Rarely, HSV-1 is responsible for a hemorrhagic encephalitis and meningitis in the brain.

The major advantage of this virus as an anti-tumor therapy is its large genome that provides the capacity to deliver a single large or multiple small transgenes [Wade-Martins *et al.*, 2001]. Indeed, this virus offers many genetic engineering possibilities and efficient methodologies for this purpose have been recently developed [Saeki *et al.*, 2001]. There are also very effective and widely available anti-HSV-1 drugs (acyclovir, ganciclovir, and valacyclovir) that can be used to curtail viral replication; similar reagents are not available with other oncolytic viruses. Moreover, the viral DNA remains in an extrachromosomal state and does not integrate into the host genome, thus limiting the possibility of recombination with the host genome and the occurrence of undesired viral mutants. HSV-1 can also infect and replicate in a wide range of cell types and can be grown to relatively high titers (up to 10^{10} pfu/ml) in culture, requirements for its use as an anti-tumor therapy. HSV-1 is endemic in humans and 50-80% of the population possesses neutralizing immunity to the virus, which might be a disadvantage. It is unclear whether a neutralizing immune response will impede the antitumor capacity of replicating oncolytic viruses such as HSV and Adenoviruses. Clinical trial data utilizing the ONYX-015 replicative adenovirus have found no correlation between baseline neutralizing antibody titers and induction of tumor response [Nemunaitis *et al.*, 2000]. Moreover, the presence of high neutralizing antibody titers did not abrogate ONYX-015 infection and/or replication within tumors. A

large number of genetically engineered HSV-1 mutants have been generated for oncolytic therapy and since three of these viruses have entered clinical trials (Table 1), data on neutralizing antibody titers for HSV should become available soon.

2. STRATEGIES TARGETING ONCOLYTIC VIRUSES TO TUMORS

Genetic modifications to the genomes of HSV-1 oncolytic vectors have been generated to preferentially target viral infection and/or replication to tumor cells versus normal cells. First, genetic alterations that eliminate critical viral protein functions essential for viral replication in normal cells, but that are non-essential in tumor cells, have been studied. Second, promoters that are highly active in tumor cells but not normal cells have been placed in front of viral genes that encode proteins essential for viral replication. Finally, genetic modifications of viral surface proteins responsible for the attachment and cell entry of HSV-1 have been designed. These different genetic alterations of the viral genome have all contributed to the differential lysis of tumor versus normal cells.

2A. Restricting Viral Replication to Tumor Cells Using Viral Gene Mutations

A number of HSV vectors have been generated with genetic modifications that eliminate viral functions necessary for replication in normal cells (Table 1). These mutations have attenuated the replicative properties of these mutant strains to permissive cells such as dividing tumor cells or cells with defects in specific cancer pathways (such as Ras).

The deletion of genes involved in HSV-mediated neurotoxicity has resulted in restricting viral replication to dividing tumor cells. The *dlspk* [Martuza *et al.*, 1991] and *hrR3* [Boviatis *et al.*, 1994; Mineta *et al.*, 1994; Yoon *et al.*, 2000] HSV vectors contain deletions of the viral thymidine kinase and *UL39* ribonucleotide reductase large subunit (ICP6) genes, respectively, which encode enzymes necessary for viral nucleotide synthesis and replication. Tumor cells have elevated levels of nucleotide precursors due to increased cell division, therefore tumor-cells will be more permissive than normal cells to the replication of these virus. A number of HSVs (R4009 [Chou *et al.*, 1990], R3616 [Chou *et al.*, 1990], R1716 [MacLean *et al.*, 1991]) have been generated that contain deletions or mutations within the $\gamma_134.5$ gene that codes for the ICP34.5 factor and these viruses have shown antitumor activity in many preclinical models [Markert *et al.*, 2001]. This dual function protein is involved in virus replication in neurons and disruption of the infected cells defense mechanisms. The loss of ICP34.5 protein function appears to restrict viral replication to transformed cells with elevated levels of Ras activity [Farassati *et al.*, 2001]. The mechanism underlying the latter involves the interaction of ICP34.5 with the Ras/ double-stranded RNA dependent protein kinase (PKR) pathway [Chiocca, 2002]. PKR, a cytosolic enzyme that is activated as a stress response following viral infection, blocks cellular and viral mRNA translation via phosphorylation of the translation initiation factor eIF-2 α . To overcome this, HSV-1 ICP34.5 interacts with protein phosphatase 1 α and possibly

other factors leading to the dephosphorylation of eIF-2 α . Activation of the Ras pathway inhibits PKR and allows HSV replication in the absence of ICP34.5. For safety issues further research in HSV oncolytic strains led to the construction of double HSV deletion mutant G207, deleted in both the γ_1 34.5 and the U_L 39 genes [Mineta *et al.*, 1995]. The oncolytic potential of this virus was tested in animal models for several tumor types such as colon [Kooby *et al.*, 1999], ovarian [Coukos *et al.*, 1999], breast [Toda *et al.*, 1998], prostate [Walker *et al.*, 1999], head and neck squamous cell cancers [Chahlavi *et al.*, 1999], melanoma [Randazzo *et al.*, 1997], and bladder [Oyama *et al.*, 2000]. Another HSV-1 that is being evaluated as an anti-tumor therapy is NV1020/R7020 [Meignier *et al.*, 1988]. This virus was initially developed to generate a herpes vaccine against HSV-1 and HSV-2 infections [Meignier *et al.*, 1988; Meignier *et al.*, 1990], although it later proved ineffective for this use. NV1020 has shown anti-tumor activity against head & neck and prostate xenografts in athymic nude mice

[Advani *et al.*, 1999; Wong *et al.*, 2001a]. While NV1020 has deletions for a number of genes that attenuate neurovirulence, including loss of one copy of the γ_1 34.5 genes, it is unknown which gene mutation(s) target the virus to tumor cells [Meignier *et al.*, 1988]. The results obtained from these pre-clinical studies led to phase I clinical trials for HSV G207 and R1716 where they have demonstrated safety by intratumoral injections [MacKie *et al.*, 2001; Markert *et al.*, 2000; Rampling *et al.*, 2000]. NV1020 has also entered phase I trials but the results are not yet published [Varghese and Rabkin, 2002].

2B. Targeting Viral Replication Using Tumor- and Tissue-Specific Promoters

A second strategy to target replication of oncolytic viruses to tumor cells is based on placing the expression of essential viral replication genes under the control of tumor- or tissue-specific promoters that are preferentially active in tumor cells (Table 1). Miyatake *et al.* modified an HSV-1

Table 1. Oncolytic HSV Vectors

Oncolytic Strains	Tumor Targeting Alteration	Permissive Cells	Cancer Targeted	Clinical Trials	Reference
DELETION TARGETING					
<i>dlspk</i>	deleted HSV-TK	replicating	pan	no	(Martuza <i>et al.</i> , 1991)
hrR3	deleted U_L 39 (ICP6)	replicating	pan	no	(Boviatsis <i>et al.</i> , 1994) (Mineta <i>et al.</i> , 1994)
R4009	deleted γ_1 34.5 (ICP34.5)	elevated ras	pan	no	(Chou <i>et al.</i> , 1990)
R3616	deleted γ_1 34.5 (ICP34.5)	elevated ras	pan	no	(Chou <i>et al.</i> , 1990)
R1716	deleted γ_1 34.5 (ICP34.5)	elevated ras	pan	phase I	(MacLean <i>et al.</i> , 1991)
G207	deleted U_L 39 and γ_1 34.5	replicating/elevated ras	pan	phase I	(Mineta <i>et al.</i> , 1995)
NV1020 (R7020)	unknown	replicating	pan	phase I	(Meignier <i>et al.</i> , 1988)
TRANSCRIPTIONAL TARGETING					
G92A	ICP4-albumin enhancer	albumin+	liver	no	(Miyatake <i>et al.</i> , 1997)
d12.CALP	ICP4-calponin promoter	calponin+	soft tissue & bone	no	(Yamamura <i>et al.</i> , 2001)
CEAICP4	ICP4-CEA promoter	CEA+	cervical, colorectal, head & neck, breast non-small cell lung, pancreas, esophageal	no	(Mullen <i>et al.</i> , 2002)
CEA γ 34.5	γ_1 34.5-CEA promoter	CEA+	same as CEAICP4	no	(Mullen <i>et al.</i> , 2002)
DF3 γ 34.5	γ_1 34.5-MUC1/DF3 promoter	MUC1/DF3+	pancreatic, lung, ovarian, breast	no	(Mullen <i>et al.</i> , 2002)
Myb34.5	γ 34.5-Bmyb promoter	replicating	pan	no	(Chung <i>et al.</i> , 1999)

mutant deleted for both *ICP4* genes to build a new virus strain, G92A, harboring an albumin enhancer/promoter-*ICP4* transgene in the *HSV-TK* locus [Miyatake *et al.*, 1997]. *ICP4* is an essential immediate early gene for HSV-1 transcription and viruses deleted for this gene cannot undergo the lytic cycle [DeLuca *et al.*, 1985; Sacks *et al.*, 1985]. Moreover, viruses deleted for the *HSV-TK* gene possess poor replicative capacity in non-dividing cells. Hence, this virus should theoretically replicate only in cycling cells expressing albumin. While this viral construct was shown to selectively replicate in hepatocellular carcinomas [Miyatake *et al.*, 1999], the presence of albumin expression in normal hepatocytes poses safety concerns for the clinical use of this virus. In a similar approach, the calponin promoter was used to drive *ICP4* expression, thereby creating a conditionally replicating HSV vector (*d12.CALP*) to target soft tissue & bone tumors [Yamamura *et al.*, 2001].

Three HSV-1 mutants were constructed to evaluate the potential use of the human carcinoembryonic antigen (CEA) and MUC1/DF3 tissue-specific promoters to regulate viral replication [Mullen *et al.*, 2002]. CEA is an oncofetal cell surface glycoprotein that is overexpressed in many tumor types. MUC1/DF3 is a glycoprotein that is overexpressed in pancreatic, lung, ovarian, and 80% of human breast cancers. The CEA*ICP4* and CEA γ 34.5 HSV-1 mutants are deleted for both copies of the endogenous *ICP4* and γ 134.5 genes, respectively, and contain a single CEA promoter-*ICP4* or γ 34.5 transgene cassette recombined into the *UL39* (*ICP6* viral ribonucleotide reductase) locus. It was found that the replication of the CEA*ICP4* virus was greatly attenuated even in several CEA-positive cell lines while CEA γ 34.5 replication was similar in CEA-positive and -negative cell lines. Possible explanations for these results include interference with the regulation of the CEA promoter by cis-acting elements in the *ICP6* gene locus and/or that the levels of *ICP4*/ γ 134.5 expressed do not support strong lytic replication. The third HSV-1 mutant created was DF3 γ 34.5. This virus is deleted for both copies of the γ 134.5 gene and contains a single MUC1/DF3 promoter- γ 134.5 transgene cassette recombined into the thymidine kinase locus. *In vitro*, DF3 γ 34.5 displayed preferential replication and cytotoxicity in infected MUC1 positive cells. Intratumoral injection of 1×10^8 plaque-forming units of DF3 γ 34.5 into tumors generated from MUC1-positive CAPAN2 human pancreatic carcinoma cells led to significant tumor growth inhibition.

The use of tissue-specific promoters to direct viral replication to a specific tumor type have been explored more extensively in oncolytic adenoviruses and the use of these promoters to regulate HSV replication could be further explored [Adachi *et al.*, 2001; Fuerer and Iggo, 2002; Nettelbeck *et al.*, 2002; Rodriguez *et al.*, 1997]. A limitation in the use of these promoters is that viral replication is mainly targeted only at a specific tumor type and this is often further restricted to only a subgroup.

To avoid this drawback, the use of promoters that are active in most tumors have been explored. All tumor cells show deregulated cell growth due to defects in the control of the G1/S phase transition during the cell cycle. The E2F-1 transcription factor activates genes controlling the G1/S

transition, including its own promoter. Furthermore, in cycling tumor cells, E2F-1 is constitutively active due to alterations in the pRb/p16INK4a/cyclin D pathway, including E2F-1 gene amplification. The Myb34.5 HSV-1 derived vector exploits this property of tumor cells. The Myb34.5 vector has the neurovirulence γ 134.5 gene under the control of the E2F responsive cellular B-*myb* promoter and was shown to induce cell lysis only in actively dividing cells [Chung *et al.*, 1999]. The same virus was successfully used in preclinical studies by intravascular injection in mice with diffuse liver metastases [Nakamura *et al.*, 2002]. Alternative tumor-specific promoters that have already been evaluated in oncolytic adenoviral vectors and which could potentially be used in HSV vectors include hypoxia-responsive promoters [Post and Van Meir, 2001 and 2003] and the telomerase promoter [Hernandez-Alcoceba *et al.*, 2002]. Another strategy currently being developed is the potential use of radiation-responsive promoters for the generation of oncolytic vectors that would replicate only after infected cells are exposed to radiation [Marples *et al.*, 2000; Scott *et al.*, 2002]. It is important to notice that such vectors do not restrict viral replication to tumor cells, per se but rather rely on the precision of radiation to target tumor. The synchronization of radiation cycles to induce efficient and controlled viral replication is an area that will require careful investigation.

While attenuated viruses carrying tumor or tissue-specific promoters can target a wide range of tumor types, there are instances where these promoters might be active in normal cells and lead to toxicity. A further major impediment to success is that in the context of a human tumor, it is likely that any of these promoters will not be active in all tumor cells and that not all cells will be infected. As a result, a subset of tumor cells will not be permissive to infection or viral-dependent cytolysis. These cells will have a selective advantage and survive leading to tumor recurrence.

2C. Targeting Viral Entry to Tumor Cells

The ability of HSV-1 to infect a wide range of cell types limits somewhat their utility as an antitumor therapy, because nontarget tissues can sequester the viruses. Retargeting these viruses preferentially to tumor cells is a two-step process involving restriction of their current host range and redirection to a new range. To achieve this, genetically engineered viral strains containing modified structural envelope proteins responsible for virus-cell interactions have been developed.

HSV-1 infection is a complex multiphase process involving the action of 11 viral envelope glycoproteins which function in cell attachment, entry, exit, and cell-to-cell spread [Roizman and Sears, 1996]. Attachment of the virus to the cell membrane is mediated by binding of the viral glycoproteins C and B to cellular heparan sulfate proteoglycans, while entrance is mediated by binding of viral glycoprotein D to the cell surface receptors HveA and HveC. HveA, a member of the tumor necrosis factor superfamily, has a restricted pattern of cellular expression [Montgomery *et al.*, 1996]. HveC, the poliovirus receptor-related protein 1, is a member of the nectin family of proteins and is widely

expressed [Geraghty *et al.*, 1998]. Altering HSV-1 host range has proved a formidable task because the viral envelope glycoproteins are multifunctional molecules that often have redundant functions.

As a first step to eliminate the natural tropism of HSV, a mutant virus (KgBpK'gC') lacking glycoprotein C and the heparan sulfate-binding domain of glycoprotein B was generated [Laquerre *et al.*, 1998b]. The KgBpK'gC' mutant HSV demonstrated an 80% reduction in binding to HSV-susceptible Vero cells compared to wild-type virus, indicating that reducing the natural HSV-1 infectivity for a wide host range was possible. However, this mutant virus was still infectious, most likely due to glycoprotein D functions. Further modifications of this virus to redirect infection to erythropoietin-receptor positive cells using glycoprotein C-erythropoietin chimeric proteins were successful although a productive infection was not established [Laquerre *et al.*, 1998a]. An alternative approach found that the attachment and essential entry functions of HSV glycoprotein D could be substituted with vesicular stomatitis virus glycoprotein G, although viral yield was drastically reduced by this pseudotyping compared to wild type glycoprotein D containing HSV [Anderson *et al.*, 2000]. A recent multifaceted approach, involving genetic modification of glycoproteins B, C, and D, was used to redirect HSV-1 entry to IL13R α 2 receptor positive cells [Zhou *et al.*, 2002]. IL13R α 2 is a brain-tumor associated receptor for Interleukin-13, and therefore viral entry via this receptor will target malignant gliomas [Debinski *et al.*, 1999; Mintz *et al.*, 2002]. In these studies, the heparan sulfate binding domain of glycoprotein B was deleted, the heparan sulfate binding domain of glycoprotein C was disrupted by replacing the amino terminal 148 amino acids with human IL-13 coding sequences, and the HveA binding site of glycoprotein D was disrupted by inserting IL-13 coding sequences at amino acid 24 to generate HSV-1 R5111 [Zhou *et al.*, 2002]. R5111 can infect J13R cells containing the IL13R α 2 receptor and lacking all other HSV-1 receptors, providing the first evidence that glycoprotein D can be modified to use another cellular protein as a receptor. It was also found that R5111 retains the capacity to interact with the HveC receptor, and therefore has the capability to infect a wide range of cell types. These results indicate that the HveA and HveC receptor interacting domains of

glycoprotein D do not overlap and can be independently disrupted. Further studies are underway to ablate the interaction of glycoprotein D with HveC. While these studies represent a step forward in specifically targeting HSV-1 entry to tumor cells, the authors have not yet addressed two important issues: (i) whether R5111 could establish a productive infection and (ii) the yield of R5111 progeny compared to that obtained with wild-type HSV-1 in permissive cells, information needed to determine if R5111 is attenuated for viral replication and progeny production. Collectively, these results indicate that the limiting factor in retargeting HSV-1 infection lies at the cell entry stage.

3. ONCOLYTIC VIRUSES IN COMBINED ANTI-TUMOR APPROACHES

While preclinical cancer models have shown the potent anti-tumor properties of oncolytic viruses, complete tumor eradications were rarely observed. Combined anti-cancer therapy approaches using oncolytic viruses in combination with chemo-, radio-, and gene therapies were tried to overcome this limitation. The rationales for multimodal therapy were manifold. Increased antitumor effects were expected with combination treatments and the different toxicity profiles of each therapy may result in enhanced efficacy without augmentation of adverse events. Also no overlapping resistance between oncolytic viruses and chemo-, radio-, and gene therapies was expected and treatment doses could potentially be reduced, thereby decreasing treatment cost, while still achieving increased therapeutic efficacy over single modality treatment.

3A. Oncolytic Viruses Combined with Chemotherapy

Combination therapy utilizing the HSV-1 R1716 and G207 vectors with several classes of chemotherapeutic agents have demonstrated additive to synergistic effects (Table 2). HSV-R1716 was tested in human non-small cell lung cancer cell (NSCLC) lines in combination with 4 agents, methotrexate (inhibits dihydrofolate reductase which is necessary for nucleotide biosynthesis), doxorubicin (topoisomerase II inhibitor that inhibits DNA synthesis), and cisplatin and mitomycin C (cause intrastrand DNA crosslinks that block DNA replication) [Toyoizumi *et al.*, 1999]. The cell killing effects appear to be dependent upon the sensitivity of the individual cell lines to the combined

Table 2. Oncolytic HSV Plus Chemotherapy Treatment

Oncolytic Strains	Chemotherapy Drug	Effect*	Tumor Type	In vitro/In vivo	Reference
R1716	methotrexate	additive	NSCLC ^b	In vitro	(Toyoizumi <i>et al.</i> , 1999)
	doxorubicin	additive		In vitro	
	cisplatin	additive		In vitro	
	mitomycin C	additive-synergistic		In vitro/In vivo	
G207	cisplatin	additive	HNSCC ^c	In vivo	(Chahlavi <i>et al.</i> , 1999)

*Effect terms as defined in original paper.

^bnon small cell lung cancer

^chead and neck squamous cell carcinoma

treatment. For example, mitomycin C plus HSV-R1716 treatment had activity that was additive in 3/5 and synergistic in 2/5 cell lines. In NCI-H460 NSCLC cells, the combined cell killing effects of cisplatin, methotrexate, or doxorubicin plus HSV-R1716 were additive while mitomycin C plus HSV-R1716 was synergistic. There was also a difference in the magnitude of the response to mitomycin C plus HSV-R1716 treatment which was additive at 3 hours and became synergistic after a 48 hour exposure. The therapeutic efficacy of combination therapy was also examined *in vivo*. In the NCI-H460 murine xenograft model, HSV-R1716 plus mitomycin C treatment reduced tumor burden in an additive manner [Toyoizumi *et al.*, 1999]. Combination therapy with cisplatin and HSV G207 in a cisplatin-sensitive head and neck cancer model resulted in a 100% cure rate compared with 42% for G207 and 14% for cisplatin single treatment groups [Chahnavi *et al.*, 1999]. These *in vivo* therapeutic effects were not associated with increased toxicity. The mechanism of synergistic activity in the combination of HSV vectors with chemotherapeutic drugs is currently unknown, although the drugs do not alter the replication or cytopathic effect of HSV-R1716 or G207, respectively.

3B. Oncolytic Viruses Combined with Radiotherapy

The combined anti-tumor effects of oncolytic viral and radiotherapies have been examined in preclinical studies because the individual cytotoxicities of these treatments are non-overlapping. Replicative HSV-1 vectors induce cytolysis while the cytotoxic effects of radiation are caused by DNA damage. The therapeutic efficacies of HSV-1 R3616, R7020, G207, and hrR3 vectors with or without radiation treatment have been evaluated in several tumor types (Table 3).

The anti-tumor efficacy of HSV-1 R3616 plus radiation therapy compared to single treatments was examined in two independent studies using human U87MG glioma xenografts in mice. In the first study, tumors xenografted in the

hindlimb were treated with large doses of radiation, virus, or radiation plus virus [Advani *et al.*, 1998] whereas the second study employed clinically relevant fractionated doses of radiation and both hindlimb and orthotopic intracranial models [Bradley *et al.*, 1999]. Enhanced synergistic antitumor activity was observed in the first and second studies, respectively.

Enhanced anti-tumor effects have also been demonstrated for HSV-1 R7020 plus radiation therapy in SQ-20b epidermoid [Advani *et al.*, 1999] and Hep3B hepatoma [Chung *et al.*, 2002] human tumor xenografts in mice. SQ-20b tumor cells are radiation resistant yet tumors treated with virus plus radiation grew 3 fold slower than the virus treatment group [Advani *et al.*, 1999]. In the hepatoma xenograft study, treatment with radiation alone, virus alone, or virus plus radiation resulted in 0%, 50%, and 88% complete tumor regression responses, respectively [Chung *et al.*, 2002]. It was also observed that HSV-1 R7020 plus radiation was highly effective for the treatment of large and small hepatoma xenografts whereas virus alone was only effective against small (<260mm³) tumors.

The *in vivo* therapeutic benefit of combining HSV-1 G207 with radiation has produced conflicting results. In two models of human and murine prostate cancer, no enhanced anti-tumor effect of HSV-1 G207 plus radiation therapy was measured compared to single treatments [Jorgensen *et al.*, 2001]. This is in sharp contrast to two other studies. Me180 human cervical carcinoma xenografts treated with low doses of radiation, G207, or radiation plus virus exhibited 1/9, 0/12, 9/12 tumor regression responses, respectively, with 5 of the tumors in the combined treatment group undergoing complete regressions [Blank *et al.*, 2002]. The antitumor effect of HSV-1 G207 was also potentiated when combined with low dose radiation using HCT-8 human colorectal cancer cells [Stanziale *et al.*, 2002]. In this system, the increased tumoricidal effect was dependent on the up-regulation of cellular ribonucleotide reductase (absent in

Table 3. Oncolytic HSV Plus Radiation Treatment

Oncolytic Strains	Effect*	Tumor Type	<i>In vitro</i> <i>In vivo</i>	Reference	Notes
R3616	>additive enhanced synergistic	cervical glioma glioma	<i>In vitro</i> <i>In vivo</i> <i>In vivo</i>	(Blank <i>et al.</i> , 2002) (Advani <i>et al.</i> , 1998) (Bradley <i>et al.</i> , 1999)	transient increase in viral yield.
NV1020 (R7020)	additive enhanced enhanced	cervical epidermoid hepatoma	<i>In vitro</i> <i>In vivo</i> <i>In vivo</i>	(Blank <i>et al.</i> , 2002) (Advani <i>et al.</i> , 1999) (Chung <i>et al.</i> , 2002)	transient increase in viral yield.
G207	>additive no effect enhanced enhanced	cervical prostate cervical colorectal	<i>In vitro</i> <i>In vivo</i> <i>In vivo</i> <i>In vivo</i>	(Blank <i>et al.</i> , 2002) (Jorgensen <i>et al.</i> , 2001) (Blank <i>et al.</i> , 2002) (Stanziale <i>et al.</i> , 2002)	increased viral burst size. Upregulation of cellular ribonucleotide reductase activity following radiation exposure.
hrR3	additive additive additive	pancreatic glioma cervical	<i>In vitro</i> <i>In vitro</i> <i>In vitro</i>	(Spear <i>et al.</i> , 2000)	viral replication not affected.

*Effect terms as defined in original paper.

G207) activity by ionizing radiation. It is difficult to compare these three studies as different tumor types were utilized and there were variations in the viral and radiation treatments. It is unclear whether modifying the experimental parameters in the prostate study would change the result as differences in tumor type and biology may effect the degree of sensitivity to viral and radiation mediated cytotoxicity. These variable results may also be due in part to differences in cellular augmentation of ribonucleotide reductase activity following radiation exposure.

The tumor cell killing effects of hrR3 in combination with radiation has only been evaluated in culture. Additive cell killing effects were observed in pancreatic, glioma and cervical tumor cells for the combination therapy compared to hrR3 or radiotherapy alone [Spear *et al.*, 2000]. Other viruses used in combination with radiation in culture include R3616, R7020, and G207. In these studies, additive to supra-additive tumor cell killing effects of cervical cancer cell lines were observed with combination therapy compared to single treatment regimens [Blank *et al.*, 2002].

The enhanced regression response of various tumor types to combined virus plus radiation therapy appears to be due in part to an augmentation of viral replication under radiation conditions. For example, increased HSV-1 R3616 replication associated with radiation exposure was measured in U87MG glioma xenografts [Advani *et al.*, 1998]. In addition, an increase in HSV-1 R7020 replication was also observed in Hep3B xenografts treated with virus plus radiation compared to virus alone [Chung *et al.*, 2002]. The effect of radiation on viral replication *in vitro* has yielded differing results. HSV-1 G207 burst size was increased 4.5-fold upon radiation treatment [Blank *et al.*, 2002] whereas hrR3 replication was not affected [Spear *et al.*, 2000]. While the mechanism(s) underlying enhanced replication of at least 3 HSV-1 vectors by radiation is unknown, this effect is not specific for HSV-

1, as it has also been observed for the CV706 Adenovirus vector [Chen *et al.*, 2001]. It is interesting to speculate that the activation of the DNA repair machinery in cells following radiation exposure may allow for greater viral replication.

These preclinical studies yielded several important findings. First, radiation did not significantly prevent HSV-1 replication and in several instances, viral yield was even augmented in the presence of radiotherapy. The radiation doses applied in these studies compounded with the relative small size of the viral genomes compared to the human genome suggest that the viral genomes do not undergo extensive damage when exposed to radiation [Chen *et al.*, 2001; Rogulski *et al.*, 2000]. Second, the virus plus radiation treatment groups did not evidence increased toxicity compared to the single treatment groups. Finally, the combination of 3 of 4 HSV-1 vectors with radiation led to a clear increase in anti-tumor efficacy in several human tumor models. These preclinical studies are encouraging and, the therapeutic efficacy of combining oncolytic viral and radiotherapies is likely to be tested in clinical trials in the near future.

3C. Oncolytic Viruses Combined with Gene Therapy

To further augment the antitumor efficacy of oncolytic HSV vectors, they have been engineered to incorporate expression cassettes for the delivery of antitumor gene therapy, including various prodrug activating (suicide) and cytokine genes (Table 4). Viral therapeutic suicide gene delivery vehicles transduce tumor cells with a suicide gene encoding an enzyme that converts an exogenously delivered non-cytotoxic prodrug into a noxious drug intratumorally [Aghi *et al.*, 2000]. Death of tumor cells is generally induced by disruption of DNA replication by the converted drug. The most studied prodrug/suicide gene system is the combination

Table 4. Oncolytic HSV Plus Gene Therapy Treatment

Oncolytic Strains	Gene	Effect	Reference
hrR3	Endogenous thymidine kinase (TK)	enhanced no effect	(Boviatisis <i>et al.</i> , 1994) (Carroll <i>et al.</i> , 1997)
HSV1yCD	Yeast cytosine deaminase	enhanced	(Nakamura <i>et al.</i> , 2001)
rRp450	CYP2B1 (cytochrome p450 2B1) and endogenous thymidine kinase	enhanced	(Chase <i>et al.</i> , 1998) (Aghi <i>et al.</i> , 1999) (Pawlik <i>et al.</i> , 2002)
R8306	Interleukin-4 (IL-4)	enhanced	(Andreansky <i>et al.</i> , 1998)
R8307	Interleukin-10 (IL-10)	no effect	(Andreansky <i>et al.</i> , 1998)
M002	Interleukin-12 (IL-12)	enhanced	(Parker <i>et al.</i> , 2000)
NV1042	Interleukin-12 (IL-12)	enhanced	(Wong <i>et al.</i> , 2001) (Bennett <i>et al.</i> , 2001)
NV1034	Granulocyte macrophage colony stimulating factor (GM-CSF)	no effect	(Wong <i>et al.</i> , 2001)
JS1-ICP34.5-/ICP47-/GM-CSF*	Granulocyte macrophage colony stimulating factor (GM-CSF)	enhanced	(Liu <i>et al.</i> , 2003)

*The JS1-ICP34.5-/ICP47-/GM-CSF HSV vector has received approval for phase I testing in the United Kingdom.

of the antiviral prodrug gancyclovir (GCV) and the herpes simplex virus thymidine kinase (HSV-TK) gene. The viral TK enzyme monophosphorylates GCV which is then converted by cellular kinases to GCV-triphosphate, an inhibitor of viral and cellular DNA synthesis. An essential feature of the TK/GCV system is the bystander effect. This is likely mediated by transfer of the toxic metabolite via gap junctions from transduced cells expressing TK to nontransduced cells, which leads to cell death. The first studies of combined oncolytic virus and prodrug/suicide gene therapy benefited from the presence of the endogenous TK gene within the HSV hrR3 vector [Boviatsis *et al.*, 1994]. In these experiments, the combination of HSV hrR3 with GCV was more efficacious than either therapy alone in the syngeneic 9L rat glioma model. In other studies the addition of GCV did not increase the anti-tumor efficiency of HSV-TK expressing oncolytic viruses (HSV-1 hrR3, Ad.wt.TK, and Ad.TK^{RC}) [Carroll *et al.*, 1997; Lambright *et al.*, 2001; Wildner and Morris, 2000a; Wildner and Morris, 2000b]. It is possible that the latter are related to the ability of GCV to inhibit viral replication, thus hindering viral intratumoral spread. Variation in the levels of gap junctions between different cell types is another potential source for variable findings [Aghi *et al.*, 1999]. In cells where viral replication is efficient and communication through gap junctions is modest (i.e. possess a weak bystander effect), the antiviral action of activated GCV might predominate over its anticancer action. In this setting, activated GCV would interfere with the viral oncolytic effect and be antagonistic. In contrast, if viral replication is relatively poor and gap junction communication is strong, the activation of GCV would increase the viral oncolytic effect. Based on this evidence, the TK gene might be best suited for gene therapies using replication-deficient viruses and as a fail-safe mechanism for viral inactivation.

Another prodrug/ suicide gene system that has been combined with oncolytic HSV vectors is the 5-fluorocytosine (5-FC) prodrug/ yeast cytosine deaminase (CD) gene [HSV1yCD, [Nakamura *et al.*, 2001]]. CD converts 5-FC into 5-fluorouracil (5-FU), a widely used chemotherapeutic agent for several tumor types. The CD/5-FC system also exhibits a bystander cell killing effect mediated by the freely diffusible 5-FU metabolite. The mechanism by which 5-FU induces cytotoxicity is not fully understood. It is converted to several metabolites that have diverse biological effects including inhibition of both cellular and viral DNA synthesis by the 5-fluorodeoxyuridylate metabolite. Consistent with this, HSV1yCD replication in HT29 human colon carcinoma cells in the presence of 5-FC was minimally reduced, less than one log order attenuation, compared to viral replication in the absence of 5-FC. Despite this, HSV1yCD plus 5-FC treatment significantly reduced the growth of MC26 mouse colon carcinoma tumors in the flank of mice and increased the survival of mice bearing diffuse liver metastases compared to the respective monotherapies [Nakamura *et al.*, 2001].

Seeking prodrug-activating genes that convert drugs into metabolites that possess primarily anticancer and not antiviral action, researchers turned to the CYP2B1 suicide gene/ cyclophosphamide (CPA) prodrug system [Aghi *et al.*,

2000]. *CYP2B1* encodes cytochrome p450 2B1 and activates the CPA and ifosfamide prodrugs into anti-cancer agents that alkylate DNA. This leads to the formation of inter- and intra-strand DNA crosslinks that result in strand breaks during DNA replication. CYP2B1/CPA gene therapy also has a strong bystander effect mediated by freely diffusible active drug metabolites. HSV rRp450 (contains endogenous TK gene) was derived from hrR3 by engineering the rat *CYP2B1* transgene in the ICP6 region [Chase *et al.*, 1998]. Initial studies examined the therapeutic effect of combining HSV pRp450 with CPA prodrug therapy in the absence of GCV. Combined treatment with HSV rRp450 and CPA exhibited augmented tumor cell killing *in vitro* and *in vivo* compared to either virus or CPA therapy alone [Aghi *et al.*, 1999; Chase *et al.*, 1998; Pawlik *et al.*, 2002]. Importantly, CPA negligibly inhibited rRp450 replication whereas its replication was reduced 2-3 logs in the presence of GCV [Pawlik *et al.*, 2002]. Surprisingly, triple treatment of rat 9L tumors with HSV rRp450 and both the CPA and GCV prodrugs led to tumor regressions whereas all other treatment combinations led to a slowing of tumor growth [Aghi *et al.*, 1999]. This effect appears to be due to a pharmacological synergism between GCV/TK and CPA/CYP2B1 gene therapies involving DNA repair. While these preclinical studies with virus plus double suicide therapy are encouraging, they utilize prodrug/suicide systems that also have antiviral activity. Therefore, the timing and dose of prodrug administration are critical parameters affecting viral replication and ultimately the therapeutic antitumor effect.

Alternative adjuvant therapy genes have been incorporated in oncolytic viruses including various cytokines that modulate the host immune response and exhibit antitumor activity. HSV vectors lacking both copies of the $\gamma_134.5$ gene and containing transgenes encoding IL-4 (R8305), IL-10 (R8307), or both subunits of IL-12 (M002) have been engineered [Andreansky *et al.*, 1998; Parker *et al.*, 2000]. IL-4 and IL-12 are antitumor cytokines that induce a host immune response against the tumor and suppress tumor angiogenesis whereas IL-10 has immunosuppressive activity. The expression of these cytokines by their respective viruses did not interfere with viral replication. Consistent with this, treatment of mice bearing intracerebral syngeneic murine GL-261 glioma tumors with R8305 (IL-4) led to prolonged survival compared to mice treated with saline or R8307 (IL-10) [Andreansky *et al.*, 1998]. A significant increase in animal survival was also observed following M002 (IL-12) treatment of mice bearing intracranial syngeneic Neuro-2A neuroblastoma murine tumors [Parker *et al.*, 2000]. The antitumor activity of R8305 (IL-4) and M002 (IL-12) were correlated with an enhanced host immune response that included infiltration of CD4⁺ and CD8⁺ T-cells and macrophages [Andreansky *et al.*, 1998; Parker *et al.*, 2000].

The oncolytic HSV NV1020/R7020 vector lacking one copy of the $\gamma_134.5$ gene has been modified to express mouse IL-12 (HSV NV1042) or GM-CSF (NV1034) [Bennett *et al.*, 2001; Wong *et al.*, 2001b]. GM-CSF regulates the development of neutrophils, macrophages, monocytes and eosinophils and is a recruiter and stimulator of dendritic cells. The expression of these exogenous cytokines did not interfere with viral replication. The antitumor efficacy of NV1042 (IL-12) was superior to that of the parental vector

NV1020 using tumors derived from murine CT26 colorectal [Bennett *et al.*, 2001] and SCC VII cutaneous squamous [Wong *et al.*, 2001b] cancer cells. The expression of GM-CSF by NV1034 did not elicit an additional therapeutic benefit in the SCC VII model and the reason(s) for this are currently unknown [Wong *et al.*, 2001b].

Another oncolytic vector that has been designed to deliver the GM-CSF transgene is JS1/ICP34.5-/ICP47-/GM-CSF [Liu *et al.*, 2003]. The JS1 HSV strain was prepared from a clinical isolate, rather than using a laboratory strain such as 17+, as it was postulated that the serial passage of viruses over time might result in reduced oncolytic potential. Indeed, the ability of JS1 to kill tumor cells *in vitro* was superior to that of the 17+ strain. To selectively target replication of JS1 to tumor cells, the two γ 134.5 genes encoding ICP34.5 were deleted. Next, the ICP47 gene was deleted. ICP47 functions to block antigen processing in HSV infected cells and therefore its deletion is anticipated to improve the stimulation of a host immune response. Consistent with this, increased class I MHC expression was observed on the surface of human cells infected with JS1/ICP34.5-/ICP47- compared to JS1/ICP34.5-. The second effect of deleting the ICP47 gene is the juxtaposition of the US11 late gene with the immediate early promoter of the deleted ICP47 gene. This results in US11 being expressed as an immediate early rather than a late gene. The rationale underlying this genetic alteration was that increased expression of US11 has been shown to enhance replication of HSV ICP34.5 mutants in tumors by blocking PKR phosphorylation. As expected, the anti-tumor effects of JS1/ICP34.5-/ICP47- were enhanced compared to JS1/ICP34.5-. To enhance the immune stimulating properties of JS1/ICP34.5-/ICP47-, the gene encoding GM-CSF was inserted into the viral genome to generate JS1/ICP34.5-/ICP47-/GM-CSF. Similar to NV1034 described above, injection of A20 lymphoma tumors growing in the flanks of immunocompetent mice with JS1/ICP34.5-/ICP47-/GM-CSF or JS1/ICP34.5-/ICP47- (lacking the GM-CSF transgene) generated similar anti-tumor effects. However, when non-injected A20 tumors growing in the contralateral flank were examined, it was found that the degree of tumor growth inhibition and the number of cured animals was greatly improved by the addition of GM-CSF. Importantly, animals cured of their A20 tumors by JS1/ICP34.5-/ICP47-/GM-CSF treatment were protected against further tumor cell challenge. Based on these promising preclinical studies, JS1/ICP34.5-/ICP47-/GM-CSF has received approval for phase I testing in the United Kingdom by direct intratumoral injection in a number of tumor types.

Overall, these results show that incorporating prodrug activating (suicide) and cytokine transgenes in the viral genome can increase the antitumor capacity of oncolytic HSV vectors. Given the heterogeneity of model systems and delivery modes used in these studies it is difficult to evaluate the relative anti-tumor potency of each virus. Expansion of these studies to a larger number of preclinical tumor models in a standardized fashion would help more precisely evaluate their potential to treat a wide spectrum of genetically and biologically diverse tumors.

CONCLUSIONS

The use of replication-competent HSV-1 vectors has become an attractive strategy to specifically target and kill tumor cells. With recent technical advances, genetic manipulations of the HSV-1 genome for the production of improved vectors has been greatly facilitated. These advances have already permitted the development of a large number of first and second-generation oncolytic HSVs. More complex vectors are currently being designed, and it will be important to develop standardized preclinical testing procedures to permit better evaluation and comparison of their relative therapeutic efficacy. The advances in targeting viral infection and replication to tumor cells have been significant, yet the anti-tumoral potency of these vectors is still insufficient, as complete tumor regression responses were not seen in preclinical models. A number of these vectors have already been used in clinical trials. These have shown limited toxicities demonstrating the overall safety of these viruses in cancer patients. Therapeutic efficacy observed in preclinical studies has not yet been observed in cancer patients, highlighting the need for new improvements. The use of oncolytic HSV-1 vectors in a combined cancer treatment regimen comprised of standard chemo- and radiotherapies as well as gene therapy holds great promise for the destruction of tumor cells and improvements in the survival of cancer patients.

ACKNOWLEDGEMENTS

We wish to acknowledge support by NIH grants CA87830 and NS41403, the C. Geyer Foundation (to E.G.V.M.), and The Brain Tumor Society (to D.E.P. and E.G.V.M.). This work is dedicated to Russell Manley who was diagnosed with terminal cancer in 2002.

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Prolonged Gene Expression and Cell Survival after Infection by a Herpes Simplex Virus Mutant Defective in the Immediate-Early Genes Encoding ICP4, ICP27, and ICP22

NAXIN WU,¹ SIMON C. WATKINS,² PRISCILLA A. SCHAFFER,³ AND NEAL A. DeLUCA^{1*}

Department of Molecular Genetics and Biochemistry¹ and Department of Cell Biology and Physiology,² University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, and Division of Molecular Genetics, Dana-Farber Cancer Institute, and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115³

Received 19 March 1996/Accepted 21 May 1996

Very early in infection, herpes simplex virus (HSV) expresses four immediate-early (IE) regulatory proteins, ICP4, ICP0, ICP22, and ICP27. The systematic inactivation of sets of the IE proteins in *cis*, and the subsequent phenotypic analysis of the resulting mutants, should provide insights into how these proteins function in the HSV life cycle and also into the specific macromolecular events that are altered or perturbed in cells infected with virus strains blocked very early in infection. This approach may also provide a rational basis to assess the efficacy and safety of HSV mutants for use in gene transfer experiments. In this study, we generated and examined the phenotype of an HSV mutant simultaneously mutated in the ICP4, ICP27, and ICP22 genes of HSV. Unlike mutants deficient in ICP4 (*d120*), ICP4 and ICP27 (*d92*), and ICP4 and ICP22 (*d96*), mutants defective in ICP4, ICP27, and ICP22 (*d95*) were visually much less toxic to Vero and human embryonic lung cells. Cells infected with *d95* at a multiplicity of infection of 10 PFU per cell retained a relatively normal morphology and expressed genes from the viral and cellular genomes for at least 3 days postinfection. The other mutant backgrounds were too toxic to allow examination of gene expression past 1 day postinfection. However, when cell survival was measured by the capacity of the infected cells to form colonies, *d95* inhibited colony formation similarly to *d92*. This apparent paradox was reconciled by the observation that host cell DNA synthesis was inhibited in cells infected with *d120*, *d92*, *d96*, and *d95*. In addition, all of the mutants exhibited pronounced and distinctive alterations in nuclear morphology, as determined by electron microscopy. The appearance of *d95*-infected cells deviated from that of uninfected cells in that large circular structures formed in the nucleus. *d95*-infected cells abundantly expressed ICP0, which accumulated in fine punctate structures in the nucleus at early times postinfection and coalesced or grew to the large circular objects that were revealed by electron microscopy. Therefore, while the abundant accumulation of ICP0 in the absence of ICP4, ICP22, and ICP27 may allow for prolonged gene expression, cell survival is impaired, in part, as a result of the inhibition of cellular DNA synthesis.

The more than 75 genes of herpes simplex virus type 1 (HSV-1) (40, 41) are expressed in a regulated and sequential manner such that three broad categories of genes, immediate-early (IE), early (E), and late (L), can be defined (26, 27). The five genes classically designated IE genes are expressed shortly after the genome arrives in the nucleus and in the absence of prior *de novo* viral protein synthesis (26, 27). Transcription of the IE genes is activated by the virion protein VP16 (3, 8), which functions as a complex on IE promoters with cellular Oct1 and other host cell proteins (19–21, 33, 46, 71). The IE genes encode the proteins infected cell polypeptide 4 (ICP4), ICP27, ICP0, ICP22, and ICP47 (10, 26, 50, 74).

ICP4, ICP0, ICP27, and ICP22 are nuclear phosphoproteins (50, 77) and possess regulatory activities which are thought to prime the cell for, and participate in, the efficient cascade of subsequent viral gene expression, DNA replication, and the production of progeny virions. ICP4 is a large multifunctional protein. It can act as a transcription factor that either represses (12, 22, 45, 48, 56) or activates (12, 16, 18, 47, 53) transcription through contacts with the general transcriptional machinery (22, 67). ICP4 is largely responsible for the transition from the

IE to E phase of viral gene expression (14, 52, 73). ICP0 will activate most test promoters in transient assays (16, 18, 47, 53) and has been found to elevate levels of viral gene expression and growth in tissue culture and in the trigeminal ganglia of mice (6, 7, 37). It also facilitates the reactivation of virus from latency in the mouse model (6, 37). ICP27 also appears to be multifunctional. Several studies have shown that it can modulate the activity of ICP4 and ICP0 (44, 54, 66), as well as the modification state of ICP4 (44, 54, 70). ICP27 has also been shown to regulate viral and cellular mRNA processing events (9, 23, 24, 42, 43, 61–63, 68). The combined activities of ICP27 contribute to efficient DNA replication and late gene expression (39, 57); however, recent studies have shown that ICP27 also significantly contributes to elevated levels of early gene expression (59, 72). The contribution of ICP27 to the elevated levels of some early proteins has provided an explanation for the requirement for ICP27 for viral DNA replication (72). ICP22 is not essential for growth in many cell types, including Vero cells, and acts to promote efficient late gene expression in a cell-type-dependent manner (65). It has also been shown to be involved in the production of a novel modified form of RNA polymerase II (55). How all four of these proteins function together to orchestrate the regulatory cascade seen in HSV-infected cells remains to be determined. Additionally, the effects of these proteins on host cell metabolism are unknown.

In the absence of ICP4, only the remaining IE proteins,

* Corresponding author. Mailing address: E1257 Biomedical Science Tower, Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261. Phone: (412) 648-9947. Fax: (412) 624-1401.

ICP6, and the L/STs (OrfP) are efficiently expressed (4, 11, 34, 35, 79). Infection of cells with ICP4 mutants promotes the rapid destruction of most cells in culture (29), whereas UV-irradiated virus (29) and mutant viruses that are also deficient in the activation function of VP16 (30) are significantly less toxic. ICP4 mutants also cause chromosomal aberrations and rapid cell death (29). Johnson and colleagues have shown that either ICP4, ICP0, ICP27, or ICP22 can significantly reduce the transformation efficiency of cultured cells to G418 resistance (30). Therefore, a tenable hypothesis is that the activities of IE proteins perturb host cell metabolism, reducing cell viability.

We have been constructing viruses that have deletions of specific IE genes and sets of IE genes in an effort to uncover possible interactions between IE proteins and to understand how HSV initially alters host cell metabolism. In addition, current virus-based strategies for using HSV as a gene transfer vehicle have not met with success. For this purpose, it is desirable to gain an understanding of how HSV affects host cell metabolism in the absence of ICP4, as well as construct mutants in the genes that contribute to the observed deleterious effects. We have previously reported viruses that contain mutations in the essential IE genes, ICP4 and ICP27, and the complementing cell lines used for their isolation and propagation (59). This report describes the consequences of the additional inactivation of the ICP22 gene. In contrast to viruses deficient in ICP4 alone or ICP4 and ICP27, viruses deficient in ICP4, ICP27, and ICP22 minimally affect cell structure. Viral and cellular gene expression continues for at least 3 days. However, cellular DNA replication and cell division are inhibited. These findings have implications for how ICP0 might affect host cell metabolism and also indicate the need to eliminate ICP0 expression if HSV is to be effectively used as a replication-defective gene transfer vehicle.

MATERIALS AND METHODS

Cells and viruses. Vero cells and human embryonic lung (HEL) cells were propagated in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum as described previously (76). The Vero-derived cell lines which provide HSV-1 IE functions in *trans*, E26 (ICP4 and ICP27), E5 (ICP4), and E8 (ICP27), were previously reported (11, 13, 59). All viruses are derived from wild-type HSV-1 strain KOS. The ICP27 and ICP22 double mutant, DMP, contains the *Sd11.2* (39) and *n199* (55) mutations, respectively. The ICP4 and ICP27 double mutant *d92* was previously described (59). *d120* (ICP4⁻) and *Sd11.2* (ICP27⁻) were previously described (11, 39). Mutant viruses were propagated and titers were determined on the appropriate cell lines complementing the defective essential viral function for productive replication. Stocks of mutant virus were tested for the quantity of viral DNA reaching the nucleus following infection and also for the expression of ICP0 by immunofluorescence as a function of multiplicity of infection (MOI) to ascertain differences in the ratio of infectious virus to PFU. The results of these tests did not indicate a significant difference in this ratio with the different viruses.

DNA preparation and Southern blot analysis. Small-scale viral DNA preparations were obtained from 2×10^5 productively infected cells. Cells were harvested when cytopathic effects were generalized. After a cycle of freezing and thawing, the suspension was sonicated and pelleted in a microcentrifuge for 30 min at 4°C. The pellet was washed with Tris-buffered saline and lysed in digestion buffer containing 0.8% sodium dodecyl sulfate (SDS) and 400 µg of proteinase K per ml for 4 h at 37°C. After phenol-chloroform extraction, the DNA was precipitated by ethanol and resuspended in TE (10 mM Tris-HCl [pH 8.0], 1 mM EDTA).

For Southern blot analysis, viral DNA samples were digested with an appropriate restriction enzyme. Digested DNA samples were subjected to electrophoresis on a 0.7% agarose gel, stained with ethidium bromide, photographed, and transferred to a nitrocellulose membrane. Prehybridization, hybridization, and washing were performed according to standard protocols (60, 69). The membrane was exposed to Amersham Hyperfilm MP.

RNA preparation and Northern (RNA) blot analysis. RNA samples were prepared by infecting 1.5×10^6 cells in 60-mm-diameter culture dishes with viruses at an MOI of 10 PFU per cell. At the appropriate time postinfection, total RNA was isolated by using the Biotex Ultraspec RNA isolation system (3a) as recommended by the manufacturer. The final RNA pellet was resuspended in

diethylpyrocarbonate-treated water, and its concentration was determined by measuring the optical density at 260 nm.

For Northern blot analysis, 5 µg of RNA was denatured in denaturing buffer (65% formamide, 8% formaldehyde, 1 mM EDTA, 20 mM morpholinepropane-sulfonic acid [MOPS], 8 mM sodium acetate) with ethidium bromide (0.5 µg/ml) at 68°C for 15 min. After being mixed with RNA loading buffer, the samples were subjected to electrophoresis on 1.3% formamide denaturing agarose gels at 35 V overnight with constant buffer circulation (28). Following electrophoresis, the gel was rinsed in water, and equal amounts of RNA samples were checked and recorded by photography on a UV transilluminator. The RNA was transferred to a nitrocellulose membrane in $20\times$ SSC (3 M sodium chloride, 0.3 M trisodium citrate), air dried, and baked at 80°C for 2 h. Prehybridization and hybridization were performed as described previously (60). To detect HSV-1 ICP0, *tk* (thymidine kinase gene), and cellular β -tubulin mRNAs, 32 P-labeled nick-translated plasmid fragments from pW3dHS8 (58) digested with *SacI* and *PstI*, pTKSS (59) digested with *EcoRI* and *BamHI*, and pRT3beta (5) digested with *PstI* were used, respectively. Quantitation of Northern blots was performed with the AMBIS 4000 radioanalysis imaging detector system.

Electrophoresis of proteins. Viral and cellular protein expression was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) of virus-infected cells labeled with 32 P or 35 S methionine. Vero or HEL cells (5×10^6) were seeded on 35-mm-diameter culture dishes. Viral infection was carried out in 0.1 ml of DMEM at an MOI of 10 PFU per cell at 37°C and 5% CO₂ with occasional rocking for 1 h. For 35 S methionine labeling, 100 µCi of 35 S methionine was added to methionine-deficient DMEM for 1 h at the indicated time. For 32 P labeling, 100 µCi of 32 P was added to phosphate-deficient DMEM at 2 to 6 h postinfection. After labeling, the cell monolayer was washed twice with Tris-buffered saline containing 500 µM *N*-*p*-tosyl-L-lysine chloromethyl ketone (TLCK). The infected cells were lysed in protein sample buffer (2% SDS, 50 mM Tris-HCl [pH 7.0], 5% β -mercaptoethanol, 0.005% bromophenol blue, 5% sucrose) and subjected to electrophoresis on an SDS-9% polyacrylamide gel. The gel was dried under vacuum and exposed to Amersham Hyperfilm.

DNA synthesis assay. To analyze DNA synthesis in cells, 1.5×10^6 cells seeded on 60-mm-diameter culture dishes were infected with virus at an MOI of 10 PFU per cell and then incubated for 1 h at 37°C and 5% CO₂ in 0.2 ml of medium. Following adsorption, the monolayers were washed twice and fresh medium was added. At the indicated time points, 100 µCi of 3 H-thymidine (New England Nuclear) was added for 3 h. After labeling, total DNA was isolated as described above except that the samples were also treated with RNase. Purified DNA was dissolved in TE, and its concentration was determined by measuring the optical density at 260 nm. The amount of 3 H incorporated was determined by liquid scintillation spectroscopy.

Colony-forming inhibition assays. Monolayers of 1.5×10^6 Vero cells were infected as described above with the indicated virus at the indicated MOI. An uninfected monolayer was maintained as a control. At 6 h postinfection, the monolayers were washed with Tris-buffered saline and trypsinized to generate single-cell suspensions. The suspensions were serially diluted and plated on 60-mm-diameter petri dishes in quadruplicate. For the efficient development of colonies, the fetal bovine serum concentration was raised to 20% and the medium was changed every 4 days. Approximately 2 weeks postplating, the colonies were fixed, stained with crystal violet, and counted. The fraction of surviving cells was represented relative to that obtained with uninfected monolayers.

Electron microscopy. Confluent monolayers of infected and uninfected cells were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (PBS) for 1 h. Following fixation, the monolayers were postfixed with 1% osmium tetroxide containing 0.1% potassium ferricyanide, dehydrated through graded alcohol, and embedded with epoxy resin. Sections (50 to 60 nm) were cut with a Reichert Ultratuc E ultramicrotome, mounted on 200-mesh grids, double stained with 2% uranyl acetate (7 min) and 1% lead citrate (3 min), and examined in a JEOL 100 CX electron microscope.

Immunofluorescence. Infected and uninfected cells were prepared on circular coverslips. Following incubation of the cultures, the culture medium was removed, and the cells were washed three times (5 min each) in PBS, fixed and permeabilized in -20°C methanol for 15 min, air dried, and then rehydrated in PBS. This procedure was followed by three washes (5 min each) in PBS containing 0.5% bovine serum albumin (BSA) and 0.15% glycine (BSA buffer). Nonspecific activity was blocked with 5% normal goat serum in BSA buffer. Subsequently, the sections were incubated for 2 h with a mouse monoclonal antibody against ICP0 (Goodwin Institute, Fla.) at a dilution of 1:100. Following incubation, the sections were washed three times in BSA buffer, and the primary antibody was revealed with a specific goat anti-mouse-Cy3.18 immunconjugate (Jackson Laboratories). To image the DNA, cells were then washed for 2 min with Hoechst 33258 (2 µg/ml) and mounted in Gelvatol (Monsanto). Image fields were collected directly at the microscope, using a 60 \times , high-numerical aperture, color-corrected oil immersion objective; a high-sensitivity, integrating three-chip Sony color camera (700 by 600 pixels); and a Coreco frame grabber board in conjunction with a Nikon FXA photomicroscope. For fluorescent images, appropriate cubes, in perfect registration, were used to collect the ICP0 and DNA signals. A further differential interference contrast image was collected to assess cellular morphology at high resolution.

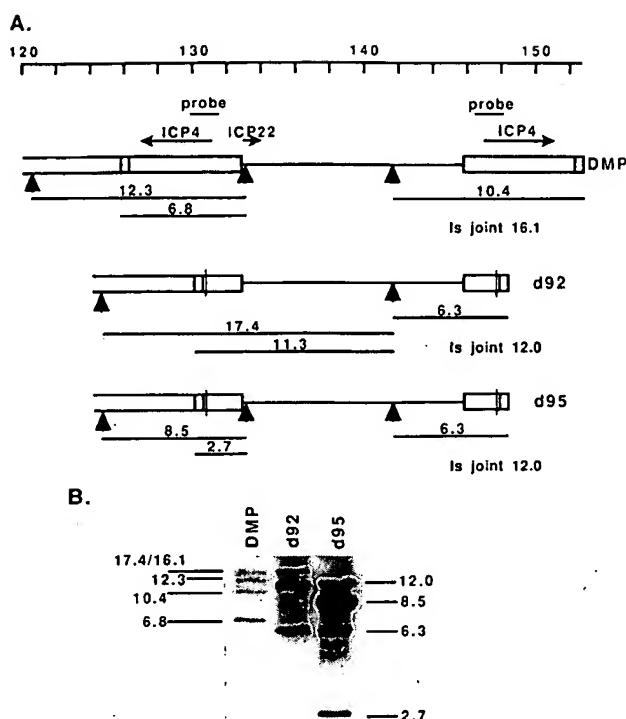


FIG. 1. (A) Schematic genome map from nucleotide 120 to the S terminus showing the locations of ICP4 and ICP22. The mutant virus, DMP, encodes a truncated ICP22 peptide of 199 amino acids by virtue of the insertion of a *HpaI* linker encoding stop codons in all three reading frames (indicated by vertical arrowheads under the ICP22 gene). The remaining arrowheads mark the natural sites of *HpaI* cleavage. Boxed regions represent the repeat region of HSV-1 viral genome. The lined region represents the unique short region of the HSV-1 genome. ICP22 and ICP4 (two copies) transcripts are represented by arrows. The *HpaI* restriction fragments and their sizes (in kilobases) are represented under the maps of the viruses. (B) Southern blot analysis. DMP, *d92*, and *d95* viral DNAs were digested by *HpaI* and subjected to electrophoresis in a 0.7% agarose gel. The fractionated DNA fragments were transferred to a nitrocellulose membrane and probed with ^{32}P -labeled 1.84-kb *Bam*HI Y fragment (probe). The sizes (in kilobases) of the bands are marked.

RESULTS

An ICP4-, ICP27-, ICP22-deficient (*d95*) virus was generated by coinfecting E26 cells (59), which supply ICP4 and ICP27, with *d92* (59) and the virus DMP. DMP is defective for ICP27 and ICP22 by virtue of the *5dl1.2* (39) and *n199* (55) alleles, respectively. As previously described, *d92* is defective for ICP4 and ICP27 by virtue of the *d120* (11) and *5dl1.2* alleles (39), respectively. Therefore, both viruses used in this cross contain the *5dl1.2* allele, ensuring that the progeny will also contain this allele. The progeny from the coinfection were plaqued on E26 cells, and individual plaques were isolated and screened for the ability to grow on ICP4- and ICP27-expressing E26 cells and not on E8 cells, which supply only ICP27. This manipulation was performed to restrict the further analysis of progeny to isolates that were genetically deficient in ICP4. Isolates that grew only on E26 cells were then screened for the incorporation of the *n199* allele by Southern blot hybridization. *n199* is marked by an *HpaI* site, which is part of a linker that specifies the stop codon conferring the ICP22⁻ phenotype.

Figure 1A shows the genome of HSV from nucleotide 120 to the S terminus in the parental orientation, the locations of the

genes for ICP4 and ICP22, and the structures of DMP, *d92*, and *d95* relative to the relevant *HpaI* restriction sites (vertical arrowheads). Also shown are the expected sizes of the *HpaI* fragments that span the ICP4 gene. The expected size of the Is joint fragment is listed for clarity. Figure 1B shows a Southern blot of the *HpaI* restriction digests of *d92*, DMP, and *d95* probed with the *Bam*HI Y fragment (Fig. 1A), demonstrating the incorporation of the *n199* insertion into the *d95* background. The sizes of the shortened fragments in the digest of *d95* relative to *d92* are consistent with the incorporation of the *n199* allele into *d95*. The sizes of the shortened fragments in the digest of *d95* relative to DMP are consistent with the incorporation of both of the 4.1-kb deletions of the ICP4 coding sequence in *d92* into *d95*. Therefore, the *HpaI* pattern of this region of *d95* is consistent with the presence of mutations in both copies of the ICP4 gene and in the ICP27 gene. The plaquing behavior of *d95* on E26, E5, and E8 cells is consistent with the presence of mutations in both copies of the ICP4 gene and the ICP27 gene.

To visualize the IE proteins synthesized in mutant-infected cells and verify the lack of ICP4, ICP27, and ICP22 synthesis, cycloheximide-treated Vero cell monolayers were infected with the indicated viruses at an MOI of 10 PFU per cell and incubated in the presence of cycloheximide for 6 h. The cycloheximide was then removed by washing the monolayer, and incubation was continued in the presence of actinomycin D and [^{35}S]methionine. Under these conditions, only the IE proteins are labeled (10, 26). While ICP4, ICP0, ICP27, and ICP22 were visible in the profiles of KOS-infected cells, the individual mutants were missing the bands corresponding to the intended mutations in the IE genes (Fig. 2A). Thus, *d95* does not synthesize ICP4, ICP27, or ICP22. The proteins synthesized in cells infected with *d120*, *5dl1.2*, and *d92* are consistent with the previously reported genotypes and phenotypes of these viruses (11, 39, 59). Also included on this gel is a sample of *d96*-infected cells. *d96* was generated by a backcross of *d95* with wild-type virus and screening for progeny that grow on E5 cells and not on Vero cells. *d96* does not synthesize ICP4 or ICP22.

To further demonstrate that *d95* does not synthesize ICP4, ICP27, or ICP22, cells infected with *d120* (ICP4⁻), *d92* (ICP4⁻ ICP27⁻), and *d95* (ICP4⁻ ICP27⁻ ICP22⁻) were metabolically labeled with $^{32}\text{P}_i$, and extracts of these cells were analyzed by SDS-PAGE. ICP27 and ICP22 are readily labeled with ^{32}P , making this approach a very good one to visualize these proteins. The resulting autoradiogram is shown in Fig. 2B. The band corresponding to ICP27 was missing in both *d95* and *d92*, while that corresponding to ICP22 was missing in *d95*. The lack of ICP22 in *d95* was also evident in the [^{35}S]methionine profile in Fig. 4. None of the mutants in Fig. 2 synthesized ICP4.

Prolonged viral and cellular gene expression in *d95*-infected cells. Levels of viral and cellular gene expression were compared in *d92*- and *d95*-infected Vero cells by SDS-PAGE analysis and Northern blot analysis. One effect that became evident early in the course of this study was that cells infected with *d120*, *d96*, and *d92* at an MOI of 10 PFU per cell could be analyzed only up to 1 day postinfection, whereas cells infected with *d95* retained a morphology more closely resembling, but not identical to, that of uninfected cells (Fig. 3). While the *d120*- and *d92*-infected cell monolayers were virtually destroyed at 2 days postinfection, the *d95* monolayer was intact (Fig. 3A). It is also interesting that there were fewer *d95*-infected than uninfected cells at this time and that many of the *d95*-infected cells consisted of two nuclei in one cytoplasmic boundary. The same general effects on toxicity and cell number were observed on HEL cells (Fig. 3B), although it was difficult to observe multinucleated cells at this level of resolution. As a

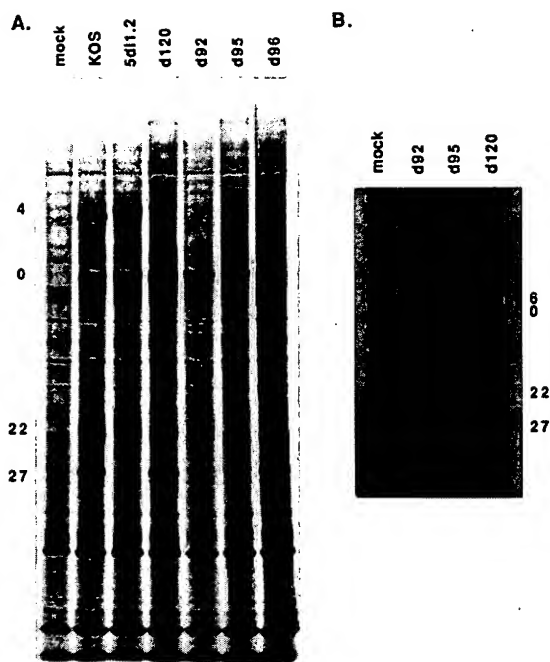


FIG. 2. IE proteins specified by wild-type and mutant viruses. (A) Cycloheximide reversal experiment. Vero cell monolayers were pretreated for 1 h by incubation in cycloheximide (100 μ g/ml)-containing medium, infected with KOS (wild type), 5dl1.2 (ICP27⁻), d120 (ICP4⁻), d92 (ICP4⁻ ICP27⁻), d95 (ICP4⁻ ICP27⁻ ICP22⁻), and d96 (ICP4⁻ ICP22⁻) at an MOI of 10 PFU per cell, and incubated in the presence of cycloheximide for 6 h. The monolayers were then washed twice and further incubated for 3 h in presence of actinomycin D (10 μ g/ml) and [³⁵S]methionine (100 μ Ci per plate). The cells were lysed in SDS sample buffer and subjected to electrophoresis on an SDS-9% polyacrylamide gel. The viral proteins ICP4, ICP0, ICP22, and ICP27 are indicated on the left. (B) Phosphoprotein synthesis in d95-, d120-, and d92-infected cells. Monolayers of Vero cells on 35-mm-diameter petri dishes were infected with d92, d95, and d120 at an MOI of 10 PFU per cell. At 2 h postinfection, the medium was replaced with phosphate-deficient medium containing 100 μ Ci of ³²P_i. Cell extracts were lysed and analyzed on an SDS-9% polyacrylamide gel. ICP6, ICP0, ICP22, and ICP27 are marked on the right.

consequence of the cytotoxicity of d120, d92, and d96, it was only possible to analyze viral and cellular gene expression in d95-infected cells past 1 day postinfection.

d92- and d95-infected Vero cells were analyzed for viral and cellular protein synthesis. Monolayers of Vero cells were infected at an MOI of 10 PFU per cell and labeled for 1 h with [³⁵S]methionine at the indicated times postinfection, and cell extracts were subjected to SDS-PAGE analysis. The resulting SDS-PAGE profile is shown in Fig. 4. Several observations can be made from these results. (i) The ICP22 band was clearly evident in the 6-, 12-, and 24-h d92 samples and not in the corresponding d95 samples. (ii) There was very little d92 sample at 2 and 3 days postinfection. This was due to loss of cells at this time and is consistent with the results shown in Fig. 3. (iii) Cellular protein synthesis in d95-infected cells remained quite high even at 3 days postinfection. This is evident by comparison with the mock-infected sample. (iv) The viral proteins ICP0 and ICP6 were abundantly expressed even at 3 days postinfection. d120 and d96 behaved like d92 with respect to the lack of longevity of protein synthesis (data not shown). This is presumably due to the toxic effects of these viruses and is consistent with the results of Johnson and colleagues (29).

To further assess gene expression in d95-infected cells, the

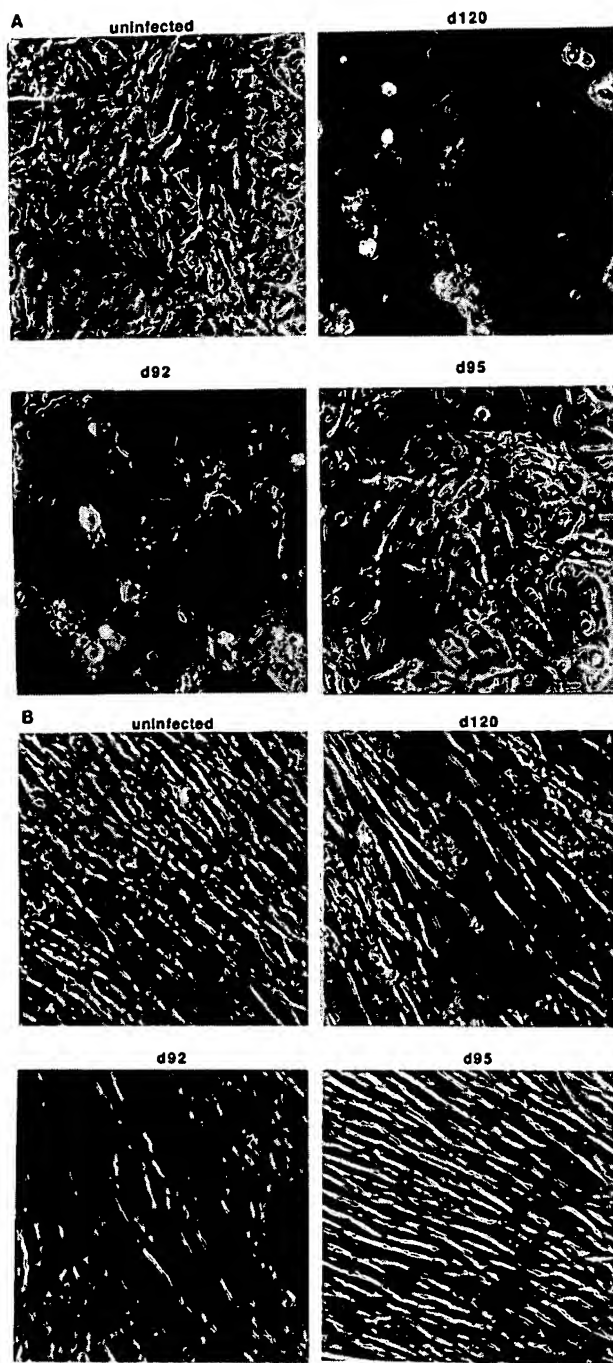


FIG. 3. Apparent cytotoxicity of d95, d120, and d92. (A) Toxicity to Vero cells. Confluent monolayer of 5×10^5 Vero cells were infected with d120, d92, and d95 at an MOI of 10 PFU per cell and incubated for 2 days. The culture were photographed through a 40 \times phase-contrast objective. (B) Toxicity to HEL cells. The assay was performed as described above except that 10^6 HEL cells were used per monolayer.

abundances of several RNA species were determined. Figure 5A shows levels of ICP0, *tk*, and cellular β -tubulin RNAs in d120-, d92-, and d95-infected cells and in uninfected cells at 6 and 24 h postinfection. Also shown is an experiment in which the levels of these transcripts were determined in uninfected

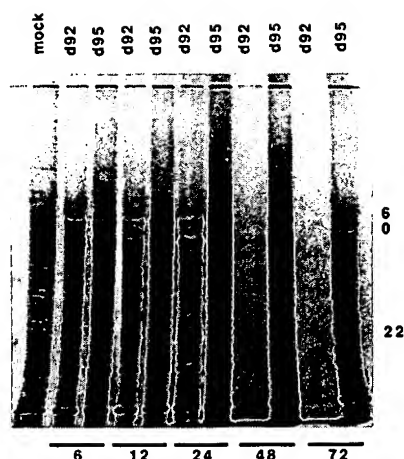


FIG. 4. Viral and cellular protein synthesis in *d92*- and *d95*-infected cells. Vero cells (5×10^5) seeded on a 35-mm-diameter petri dish were infected with *d92* and *d95* at an MOI of 10 PFU per cell. At 6, 12, 24, 48, and 72 h postinfection, cells were pulse-labeled for 1 h by incubation in presence of 100 μ Ci of 35 S-labeled methionine per ml. After the labeling period, the cells were solubilized in SDS sample buffer and electrophoresed on an SDS-9% polyacrylamide gel. The positions of ICP0, ICP6, and ICP22 are indicated on the right.

and *d95*-infected cells at 24, 48, and 72 h postinfection (Fig. 5B). It should be noted that while ICP0 is abundantly transcribed in the absence of ICP4, *tk* is not. The levels of *tk* seen in the absence of ICP4 are approximately 2 to 4% of those seen in the presence of ICP4 (28). Consistent with previous studies, ICP0 RNA was slightly increased in size in *d92*-infected cells relative to *d120*-infected cells, and the abundance of *tk* RNA was less in *d92*-infected cells than in *d120*-infected cells, at 6 h postinfection (59). Curiously, deletion of ICP22 from the *d92* background suppressed these effects. The effect on ICP0 RNA was less evident at 24 h postinfection, and the effect on *tk* RNA was no longer observed. Consistent with the labeling of cellular

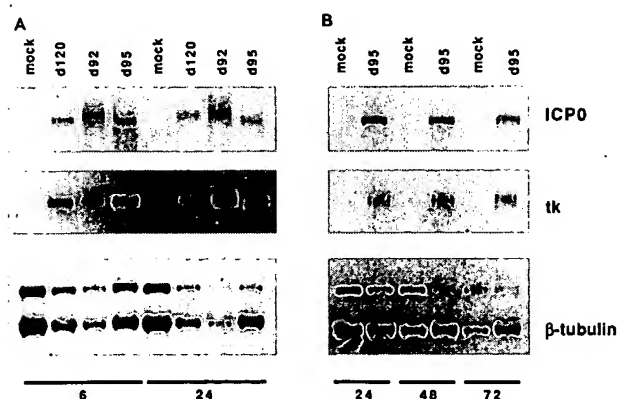


FIG. 5. Accumulation of ICP0, *tk*, and β -tubulin RNAs in *d120*-, *d92*-, and *d95*-infected Vero cells. Vero cells were mock infected or infected with *d120*, *d92*, and *d95* at an MOI of 10 PFU per cell. At 6 and 24 h postinfection, total RNA was isolated and 5 μ g of each sample was subjected to Northern blot analysis. ICP0, *tk*, and β -tubulin RNAs were probed with the probes described in Materials and Methods (A). Uninfected and *d95*-infected Vero cells (MOI of 10) incubated for 1, 2, and 3 days were analyzed in a similar manner (B). Because of alternative polyadenylation site usage, two β -tubulin mRNAs were detected. The 1.8-kb species results from utilization of the proximal poly(A) site, and the 2.6-kb species results from utilization of the distal poly(A) site.

proteins in the SDS-PAGE profile in Fig. 4, the abundance of β -tubulin RNA was greatest in the *d95*-infected cells, being comparable to that in uninfected cells. Therefore, despite the equal loading of total cellular RNA as determined spectrophotometrically and by the ethidium bromide staining patterns of the rRNA, β -tubulin RNA was less abundant in *d120*- and *d92*-infected cells than in *d95*-infected cells. This finding implies that the stability or the transcription of these messages is reduced as a consequence of the genes expressed in *d120* and *d92* and that the further removal of ICP22 relieved this effect. The abundances of all three of the messages in *d95*-infected cells remained relatively unchanged up to 3 days postinfection (Fig. 5B). However, after 3 to 4 days at an MOI of 10, the monolayer lost its integrity; consequently, these times were not analyzed. The same patterns of expression of ICP0, *tk*, and β -tubulin RNAs seen in Vero cells were also seen in HEL cells (data not shown).

Quantitative analysis of the β -tubulin RNA in Fig. 5 is shown in Fig. 6. At 6 and 24 h postinfection, the levels of β -tubulin RNA were reduced in *d120*-, *d92*-, and *d95*-infected cells, with the least reduction seen in *d95*-infected cells (Fig. 6A). While the levels of β -tubulin RNA declined in uninfected cells over the course of 3 days, β -tubulin RNA levels in *d95*-infected cells remained constant over this time period (Fig. 6B), as did the levels of ICP0 and *tk* RNAs over this time interval (Fig. 5B). The simplest interpretation of the data is that HSV proteins expressed from the *d95* genome, including ICP0, allow for transcription to continue at a constant rate over the 3-day period.

It is clear from Fig. 5 that β -tubulin RNA is present in two species. This has been previously reported and results from the use of alternative polyadenylation signals (36). Figures 6C and D show the ratios of the low (1.8-kb)- to high (2.6-kb)-molecular-size species, indicative of the relative usage of the proximal and distal poly(A) sites. This usage changed as a function of the viral genetic background by 1 day postinfection. Utilization of the proximal signal increased when ICP27 was deleted, as demonstrated by the increase in the low/high ratio in *d92*- and *d95*-infected cells relative to *d120*- and mock-infected cells at 24 h postinfection (Fig. 6C). The usage of the proximal poly(A) site became more pronounced by 2 and 3 days postinfection in *d95*-infected cells relative to uninfected cells (Fig. 6D). Therefore, the HSV proteins expressed from the *d95* genome, including ICP0, result in the alteration of 3' processing relative to uninfected cells in the case of β -tubulin RNA. It is also possible that the *d95* background results in an altered relative stability of the two processed forms of the message. Apparently, the added expression of ICP27 results in the alteration of poly(A) site usage back to a proportion observed in uninfected cells.

Inhibition of cell division and DNA replication in *d95*-infected cells. While cells infected with *d95* do not exhibit rapid rounding up and detachment from the monolayer and continue to express viral genes for at least 3 days, they do not increase in number. This is evident to some degree in Fig. 3 and is represented quantitatively in Fig. 7A. While uninfected Vero cells in a monolayer increased in number over 2 days, *d95*-infected cells did not. Rather, a marginal decrease in cell number was evident. Therefore, it appears the growth potential of *d95*-infected cells was inhibited.

To assess the growth potential of *d95*-infected cells, two experiments were performed. The first involved infecting monolayers of Vero cells with *d120*, *d92*, and *d95* at several multiplicities, followed by trypsinization and plating to measure CFU (Fig. 7B). The second measured incorporation of [3 H]thymidine into infected cell DNA (Fig. 7C and D). In the

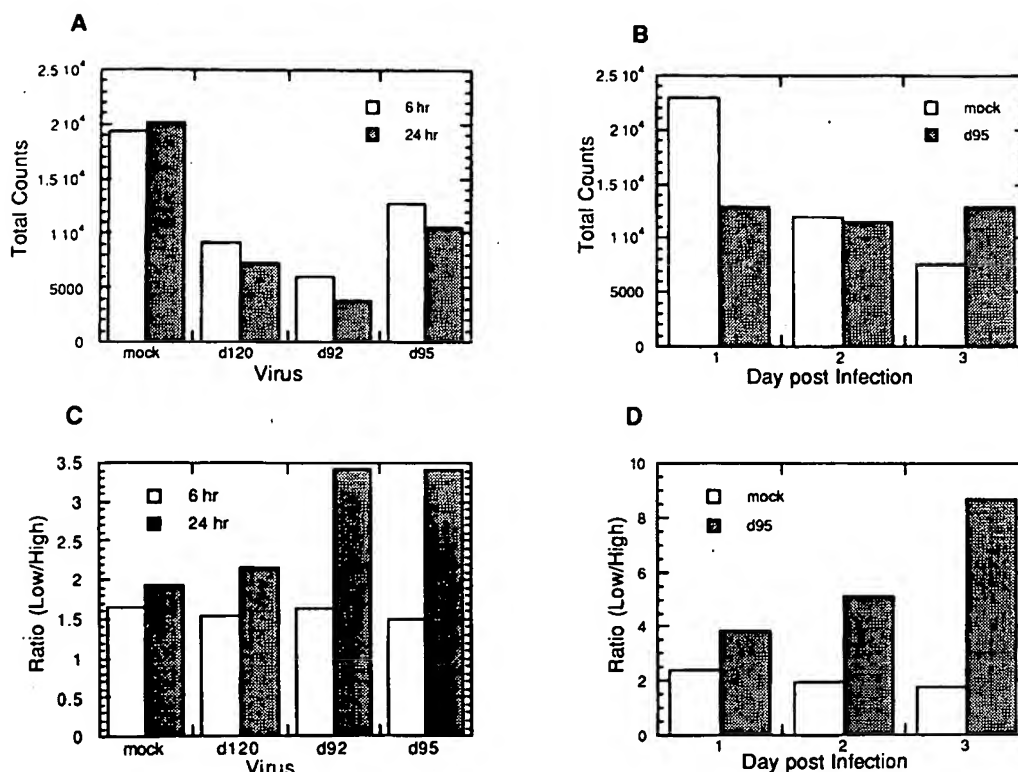


FIG. 6. Quantitation of mRNA accumulation and poly(A) site usage of cellular β -tubulin. (A) Counts of ^{32}P hybridizing to the 1.8- and 2.6-kb species in Fig. 5A were determined as described in Materials and Methods. (B) Counts of ^{32}P hybridizing to the 1.8- and 2.6-kb species in Fig. 5B were determined as described in Materials and Methods. (C) The ratios of the net counts of the 1.8-kb over 2.6-kb species in Fig. 5A. (D) The ratios of the net counts of the 1.8-kb over 2.6-kb species in Fig. 5B.

colony-forming assay, *d92* inhibited cell viability less than *d120*. Interestingly, *d95* was only marginally less inhibitory than *d92*, despite the dramatically different appearance of *d92*- and *d95*-infected cells shown in Fig. 3. Figure 7B also shows the probability of the cells not being infected following inoculation at a given MOI. The survival curves indicate that up to an MOI of 3, a single PFU is very efficient in inhibiting colony formation. At an MOI of 10, survival is greater than would be expected from the pattern seen at the lower MOIs. This finding indicates that the inhibitory effects may be saturable or that there may be subpopulations of cells that are less susceptible to the inhibitory effects of IE proteins. In summary, all of these viruses had a significant inhibitory effect on colony-forming ability, indicating that fundamental cellular processes required for cells to form colonies are perturbed by HSV, even when ICP4, ICP27, and ICP22 are not expressed.

To study this effect in greater detail, we determined if cellular DNA synthesis was inhibited in *d95*-infected cells. Accordingly, Vero and HEL cells were infected with *d95* at an MOI of 10 PFU per cell. At 1, 2, 3, and 4 days postinfection, *d95*-infected and uninfected cells were labeled for 3 h with [^3H]thymidine. Following the labeling period, DNA from the cells was isolated and the amount of ^3H incorporated per microgram of DNA was determined. As is evident in Fig. 7C and D, *d95* infection significantly inhibited cellular DNA replication in both Vero and HEL cells, respectively. The reduction in labeling of uninfected cells at 3 and 4 days postinfection is consistent with results of Fig. 7A, probably reflecting contact inhibition. To determine the level of DNA synthesis as a func-

tion of the other IE mutant backgrounds, Vero cells were infected with *d120*, *d92*, *d95*, and *d96* at an MOI of 10 PFU per cell and labeled with [^3H]thymidine from 21 to 24 h postinfection. As described above, DNA was isolated and the quantity of ^3H incorporated per microgram of DNA was determined. The resulting levels of incorporation of [^3H]thymidine relative to that in uninfected cells were 25% for *d120*, 12% for *d92*, 13% for *d95*, and 25% for *d96*. These results suggest that the cellular environment in all of these mutant backgrounds is incompatible with uninfected levels of cellular DNA synthesis.

Perturbation of nuclear structure by HSV IE gene mutants. It has been known for some time that ICP4 mutants of HSV have a deleterious effect on cellular morphology and chromatin structure (29, 49). To determine the contributions of the IE proteins to morphological changes in the cell, Vero cells were infected at an MOI of 10 PFU per cell with *d120*, *d92*, *d95*, and *d96* and processed for electron microscopy at 24 h postinfection. Figure 8 shows that all of the mutants elicit changes or the formation of novel structures relative to uninfected cells. Normal cell morphology is shown in Fig. 8A. Infection of cells with *d120* (Fig. 8B) resulted in the accumulation of small dense intranuclear granules. The nucleus commonly had a highly convoluted profile, and frequently a series of large proteinaceous cytoplasmic bodies was seen. *d96* (Fig. 8F) had morphologic sequelae similar to those for *d120* except that the small dense intranuclear granules were absent. In *d92*-infected cells (Fig. 8C), no large cytoplasmic bodies were seen; rather, these structures were confined to the nucleus. The *d92*-infected cells also showed small nuclear granules (as seen in the *d120*-

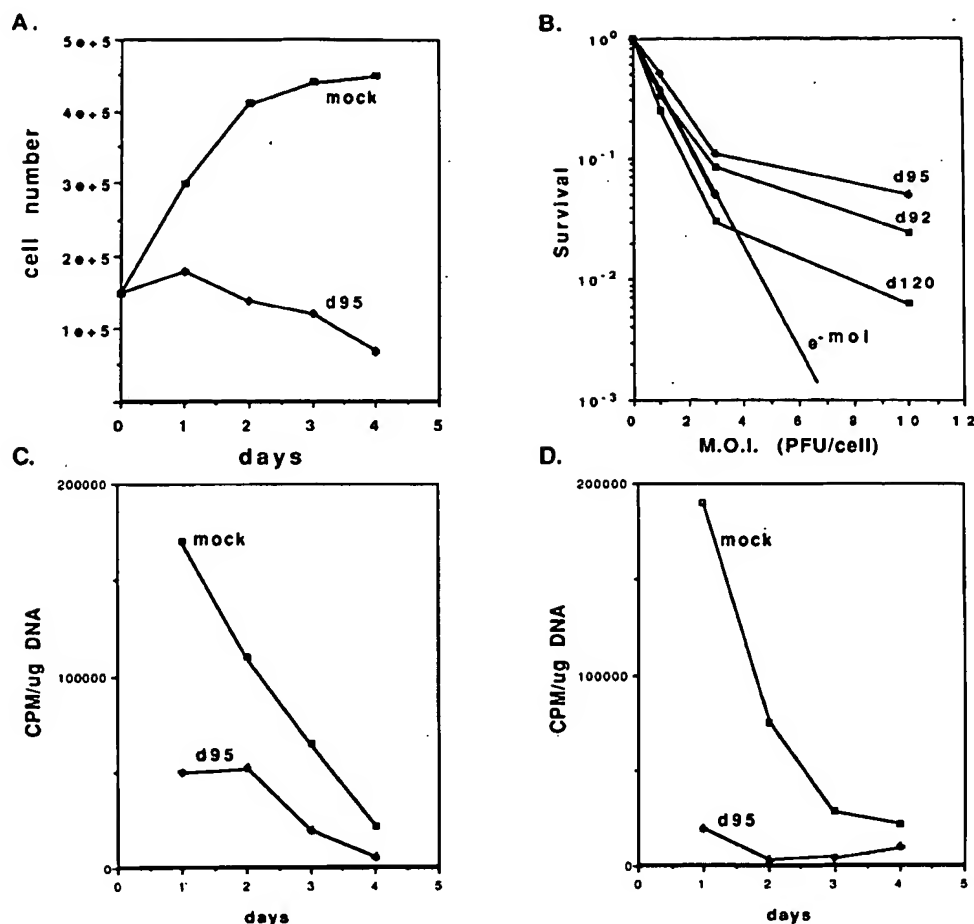


FIG. 7. Growth potential of *d95*-infected cells. (A) Cell number as a function of time postinfection with *d95*. Monolayers of Vero cells were mock infected or infected with *d95* at an MOI of 10 PFU per cell. At 1, 2, 3, and 4 days postinfection, the monolayers were trypsinized and the cells were counted in a hemocytometer. Shown are the cell counts per milliliter from a 3-ml suspension. (B) Colony inhibition assay. Confluent monolayers of Vero cells were mock infected or infected with the indicated viruses and MOIs. The monolayers were trypsinized at 6 h postinfection and plated for CFU as described in Materials and Methods. Shown are the surviving fraction of infected cells relative to uninfected cells. Also shown is the probability of a cell not becoming infected as a function of MOI ($e^{-\text{moi}}$). (C) Incorporation of [^3H]thymidine into Vero cell DNA as a function of time after infection with *d95*. Monolayers of Vero cells were mock infected or infected with *d95* at an MOI of 10 PFU per cell. At 1, 2, 3, and 4 days postinfection, the cultures were labeled for 3 h with [^3H]thymidine, and the cellular DNA was extracted, quantified, and counted for ^3H as described in Materials and Methods. (D) Incorporation of [^3H]thymidine into HEL cell DNA as a function of time after infection with *d95*. This experiment was performed like that in panel C except that HEL cells were used in place of Vero cells.

infected cells); however, they commonly appeared as larger condensed structures about the large nuclear bodies (Fig. 8C and D). *d95*-infected cells appeared to be the least affected; however, numerous large and regularly shaped nuclear inclusions were evident in these cells (Fig. 8E). These inclusions were noticeably absent from uninfected cells and are reminiscent of the nuclear bodies seen in *d92*-infected cells.

ICP0 is abundantly synthesized in *d95*-infected cells (Fig. 4). To address the possibility that ICP0 is present in the dense nuclear bodies in *d95*-infected cells, *d95*-infected cells were stained with ICP0 antibody and processed for immunofluorescence microscopy. Shown in Fig. 9 are fluorescent images of *d95*-infected cells (MOI of 10 PFU per cell) stained with a monoclonal antibody to ICP0 at 6, 12, 24, and 48 h postinfection. The insets show an enlargement of a nucleus from the larger field. As previously observed (31, 81, 82), ICP0 accumulated in fine punctate structures at early times (6 h) postinfection (Fig. 8A). Subsequently, the continued accumulation of ICP0 in the nucleus resulted in the formation of fewer but much larger ICP0-containing bodies (Fig. 8B to D). The per-

turbation of nuclear structure and number often seen with IE mutants is evident in Fig. 8D and E. Figure 8E shows the same field as in Fig. 8D, but the DNA has been specifically stained with Hoescht dye. From this micrograph, it is clear that the ICP0-containing structures do not contain DNA. Figure 8F is a differential interference image of the same cell as in Fig. 8D and E. The ICP0-containing structures are easily resolved in this image. Thus, in the absence of ICP4, ICP27, and ICP22, ICP0 accumulated to very high levels in the nucleus and localized to dense, spherical bodies. The formation of these structures represents the only obvious deviation from the morphology of uninfected cell nuclei.

DISCUSSION

The ability of virus-encoded functions to rapidly usurp host cell metabolic mechanisms along with the function of viral systems enables HSV to express its 75 to 80 genes, replicate its genome, and assemble progeny within 6 h of infection in susceptible cells. While significant progress has been made toward

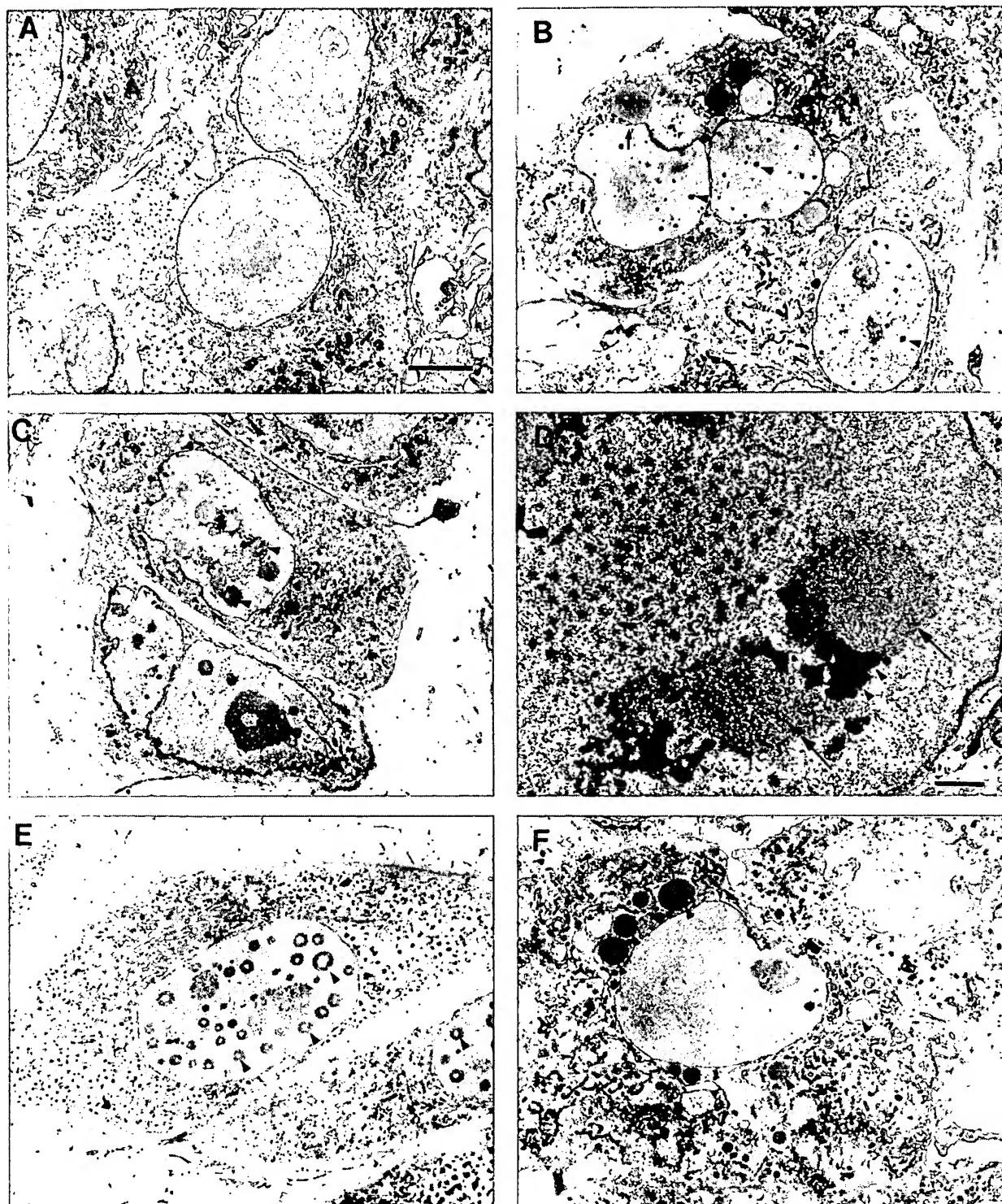


FIG. 8. Electron micrographs of Vero cells after infection with different IE mutants. Eighty percent confluent monolayers of Vero cells were uninfected or infected with *d120*, *d92*, *d96*, or *d95* at an MOI of 10 PFU/ml. At 24 h postinfection, the monolayers were processed for electron microscopy as described in Materials and Methods. (A) Uninfected cells show a typical cell and nuclear morphology, with large prominent nucleoli and thin heterochromatin (bar = 5 μ m). (B) Cells infected with *d120*. Small intranuclear (arrowheads) and large cytoplasmic (arrows) inclusions are apparent. Magnification as in panel A. (C and D) In cells infected with *d92*, at low power (C), large (arrowheads) and small intranuclear particles are apparent. At high power (D), the small particles are seen to exist both as small stellate granules and as structures condensed about the larger inclusions (arrows). C, magnification as in panel A; D, bar = 0.5 μ m. (E) Cells infected with *d95*. Large abundant nuclear inclusions are seen (arrowheads). Serial sectioning shows these structures to be spherical (not shown). Magnification as in panel A. (F) Cells infected with *d96*. The nucleus appears similar to that seen in control cells (A); however, abundant, large cytoplasmic inclusions are apparent (arrowheads). Magnification as in panel A.

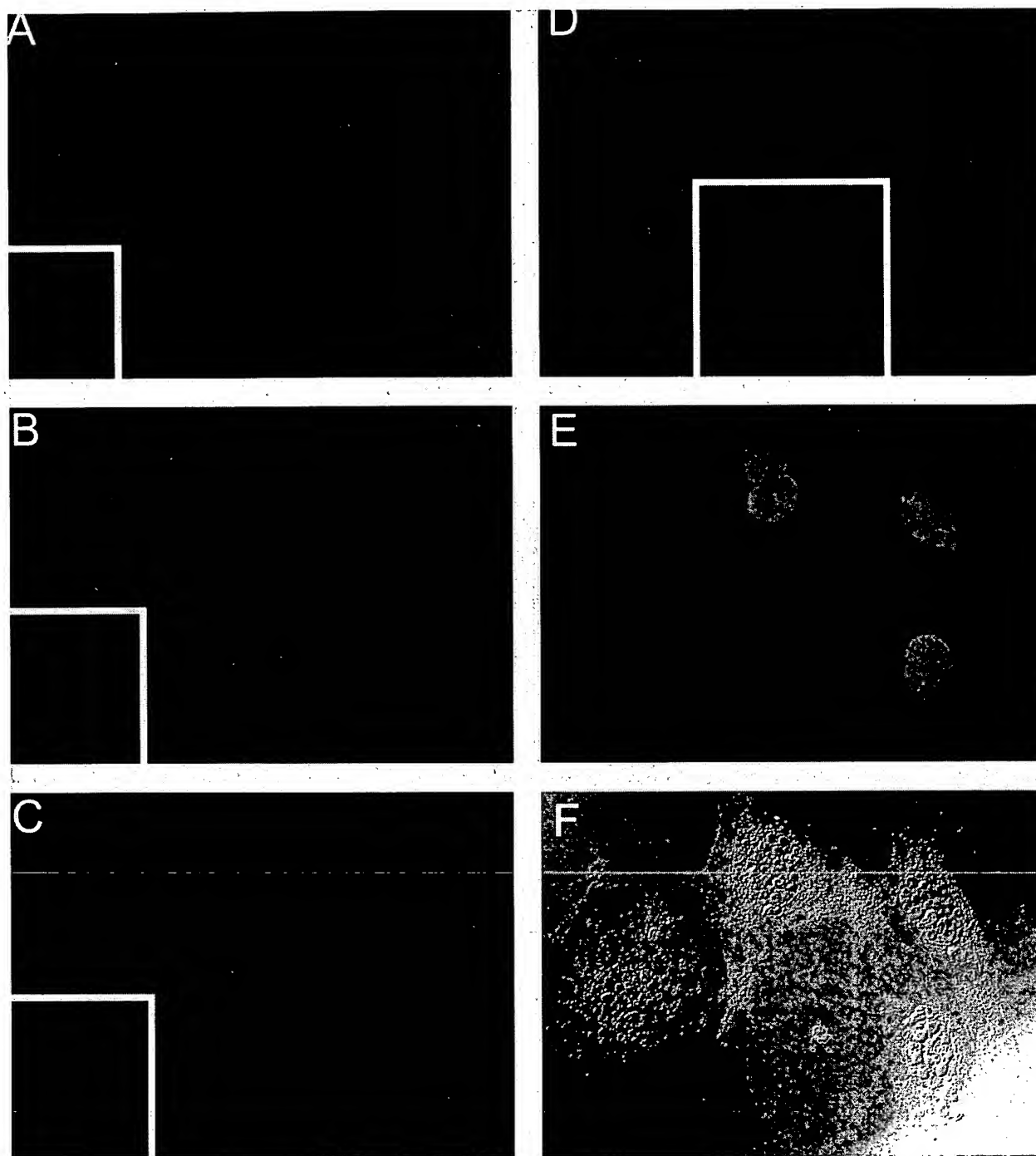


FIG. 9. Localization of ICP0 in *d95*-infected cells. Eighty percent confluent monolayers of Vero cells were infected with *d95* at an MOI of 10 PFU/ml and processed for immunofluorescence at 6 (A), 12 (B), 24 (C), and 48 (D to F) h postinfection, using a monoclonal antibody to ICP0 as described in Materials and Methods. At early time points, the ICP0-containing nuclear inclusions appear small and granular, with a high nucleoplasmic staining. With time, the size of the inclusions increases dramatically, while the nucleoplasmic background staining decreases. (E) When the DNA within the nucleus is counterstained with Hoescht dye, it is apparent that the ICP0-staining granules contain no DNA. (F) When the cells are examined by differential interference contrast microscopy, while the cellular cytoplasmic profile appears normal, the multiple nuclear inclusions are apparent. Panels D to F are the same field.

the understanding of viral functions, less light has been shed on how these functions perturb or alter host cell processes.

It has been known for some time that infection of cells with viruses deficient in ICP4 results in rapid cell death (29) accom-

panied by chromosomal damage (29, 49) and alterations in nuclear structures. In the absence of ICP4, at least five other gene products, ICP27, ICP0, ICP22, ICP47, ICP6, and OrfP, are efficiently synthesized (4, 11, 34, 35, 79). Many of these

proteins have been shown to have activities that may perturb host cell functions. Indeed, it has been demonstrated that ICP27, ICP0, and ICP22 reduce the efficiency with which cells are transformed with a selectable marker, implying that they reduce cell viability (30).

In this study, the effects of a series of HSV mutants defective in ICP4, ICP22, and ICP27 on gene expression, host cell viability, cellular DNA replication, cell division, and nuclear ultrastructure were studied. These experiments revealed that infection of cells with a mutant defective in ICP4, ICP22, and ICP27 (*d95*) resulted in prolonged cell viability, as measured by viral and cellular gene expression (Fig. 4 and 5) and overall cell morphology relative to *d120*- and *d92*-infected cells. The colony-forming capacity, or the capacity of *d95*-infected cells to multiply, was not significantly greater than that of *d92*-infected cells (Fig. 7B), despite the large difference observed in the appearance of *d92*- and *d95*-infected monolayers (Fig. 3). Additionally, *d95* had the least effect on nuclear morphology of all of the mutants tested (Fig. 8). The only deviation from uninfected cell morphology was the accumulation of dense, spherical structures in the nucleus, which were subsequently shown to contain ICP0 (Fig. 9).

Gene expression in mutant virus-infected cells. In HSV-1-infected cells, the transcription of viral and cellular genes is carried out by host RNA polymerase II (1). ICP4 has a direct effect on transcription, and ICP0, ICP27, and ICP22 have all been implicated in modulating transcription. ICP0 is a promiscuous transactivator which has been reported to transactivate a variety of viral and cellular promoters (16, 18, 47, 53). ICP0 is abundantly expressed in *d95*-infected Vero cells. The level of ICP0 transcript and the rate of ICP0 protein synthesis were relatively constant from days 1 to 3 postinfection. In addition, the accumulation of a cellular message was maintained at a high level throughout this time period. It is possible that the expression of ICP0 in the absence of the deleterious effects of other IE genes allows for the observed prolonged gene expression.

The striking difference in cell morphology and survival was observed when ICP22 was inactivated. Mutants in ICP22 show reduced transcription and expression of certain viral genes (55, 65). ICP22 also induces a novel phosphorylated form of cellular RNA polymerase II (55). These observations are all consistent with transcriptional effects, with the presence of ICP22 correlating with more efficient viral transcription. By virtue of the prolonged accumulation of the products of viral and cellular genes, we apparently see more efficient gene expression in the absence of ICP22. Previous studies of ICP22, including some using the same ICP22 allele in *d95*, have all focused on the effect of ICP22 in an otherwise wild-type background. It is possible that the prolonged gene expression that is observed in this study with *d95* compared with *d92* is related not to a direct effect of ICP22 on gene expression but rather to some other deleterious effect that is relieved by the inactivation of ICP22. Comparison of the activities of the transcriptional apparatus in *d92*- and *d95*-infected cells should shed some light on this question.

ICP27 has been shown to affect two aspects of mRNA processing: splicing and poly(A) site usage (23, 42, 43, 61–63). Effects on mRNA structure were observed in two cases in this study. The first is that the *tk* and ICP0 messages were slightly larger and appeared more heterogeneous when synthesized in the *d92* (ICP4[−] ICP27[−]) background compared with the *d120* (ICP4[−]) background. This effect was suppressed when ICP22 was mutated (*d95*). The second is that the deletion of ICP27 from the *d120* background resulted in a change in the relative utilization of the β -tubulin poly(A) sites (Fig. 5 and 6) at 24 h

postinfection. The further deletion of ICP22 had little or no effect on poly(A) site utilization; however, the effect became more pronounced because of the extended life span of the infected cells. This observation also demonstrates that in the *d95* background, the poly(A) site usage may be different from that in uninfected cells. This effect is unexpected since none of the proteins expressed in this background is known to have an effect on poly(A) site usage. These observations on viral and cellular messages, the former in particular, are consistent with effects of ICP27 on poly(A) site selection (42, 43). The effect on ICP0 and *tk* messages also suggests a functional interaction between ICP27 and ICP22. Deleting ICP27 (in *d92*) resulted in altered mRNA structure relative to *d120*, and the further inactivation of ICP22 in *d95* suppressed this effect.

Cytotoxicity and the inhibition of DNA synthesis. In the absence of ICP4, ICP27, and ICP22, the infected cell survives longer than cells infected with an ICP4[−] ICP27[−] mutant, as is clear from microscopic examination of cells and studies on cell and viral gene expression. Despite this, the ability of *d95*-infected cells to proliferate and form colonies was impaired. This impairment is due at least in part to factors contributing to the inhibition of cell DNA synthesis. Presumably, the proteins synthesized in the *d95* background (ICP6, ICP0, ICP47, and OrfP) are responsible for this effect. ICP47, a cytoplasmic protein that has been shown to affect the processing of major histocompatibility complex class I molecules (80), is probably not responsible for this effect. ICP6 is also probably not involved since a virus deleted for ICP6, ICP4, and ICP27 still inhibits cell DNA synthesis (unpublished data). This leaves OrfP and ICP0 as potential candidates for this effect. Additionally, ICP0 may induce changes in cellular gene expression that result in the inhibition of cellular DNA synthesis or cell cycle arrest. It is also possible that the virion-associated host cell shutoff function, UL41 (53a), also contributes to the shutoff of host cell DNA synthesis. However, two observations suggest that this is at most a minor component of the shutoff of DNA synthesis: (i) at the MOIs used in this study, *d95* did not efficiently shut off host cell protein synthesis (Fig. 3), and (ii) a virus lacking ICP4, ICP27, and UL41 still shuts off host cell DNA synthesis (data not shown).

ICP0 clearly alters the nuclear ultrastructure and potentially the compartmentalization of nuclear proteins involved in cell cycle regulation. In cells infected with wild-type virus (31) or an ICP4[−] ICP27[−] mutant (*d92* [82]), ICP0 accumulates in many small punctate intranuclear structures. Everett and Maul have shown that these structures are coincident with PML-containing structures (17, 38), also known as ND10, PODs, or Kr bodies (2, 15, 32, 75, 78). The function of these structures is unknown but is thought to be involved in the proliferative or differentiation state of the cell (15, 32, 75). In a wild-type virus background, ICP0 dissociates these structures late in infection and itself becomes more diffusely localized throughout the cell (17, 38). In *d95*-infected cells, ICP0 accumulates over the course of 3 days (Fig. 3). Early in infection, its localization is similar to that seen previously, i.e., an abundance of small punctate structures is evident over a diffuse background. However, with time, ICP0 accumulates into increasingly large structures, which are clearly visible by light microscopy. These structures do not contain significant quantities of DNA. One possibility is that they represent inclusion bodies which have nucleated at the ND10 structures. From previous results, it is likely that ND10 structures are disturbed, and it is also possible that cellular molecules, which interact with ICP0, are sequestered into these inclusion bodies. Further studies on the compositions of these structures and the cellular proteins that they may contain are in progress. A remaining formal possibility is

that a truncated ICP22 molecule is synthesized as a function of *n199* allele, and this peptide is involved in the phenotype of *d95*. However, the *n199* allele imparts a growth restriction similar to that seen with complete deletions of the ICP22 gene (51). *d95* titers are reduced 5- to 10-fold relative to *d92* titers (data not shown). Also, the region of ICP22 that is most conserved among the herpesviruses (25, 51) is excluded by virtue of the *n199* mutation. An additional unlikely possibility is that a fortuitous gain-of-function mutation contributes to the phenotype of *d95*.

In the absence of ICP4, ICP27, and ICP22, cell DNA synthesis was inhibited and ICP0 accumulated to very high levels in discrete structures in the nucleus. The formation of large nuclear inclusions correlated with the absence of ICP27. This finding may reflect previous observations that the expression of ICP27 in the absence of ICP4 results in the cytoplasmic localization of ICP0 (81, 82). Also of note is the observation that the expression of ICP22 results in the formation of fine granular structures in the nucleus. In the presence of nuclear ICP0, as in *d92*, these appeared to coalesce around the ICP0-containing bodies (Fig. 8D). This finding raises the possibility that molecules present as a function of ICP22 interact with ICP0. Further studies on the identities and compositions of the ICP22-dependent structures will be necessary to address this question.

The observations described in this study reflect some of the effects of the IE proteins on host cell metabolism. Presumably some of these changes occur early in the viral infection and prime the infected cell for productive viral infection. It is also possible that some of the observed effects are exaggerated or are a consequence of IE protein overexpression. In either case, these results also bear on the use of IE deletion mutants of HSV for use as gene transfer vehicles. While viral backgrounds such as *d95* may have utility in some cases or in certain cell types, it is highly likely that all of the regulatory IE genes are deleterious to host cell survival and that they will all have to be deleted if HSV is to be generally used as a vector.

ACKNOWLEDGMENTS

This work was supported by NIH grants AI30612, DK44935, and CA20260.

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ANALYSIS OF NUCLEOTIDE SEQUENCE VARIATIONS IN HERPES SIMPLEX VIRUS TYPES 1 AND 2, AND VARICELLA-ZOSTER VIRUS

A. CHIBA¹, T. SUZUTANI^{1*}, M. SAIJO², S. KOYANO^{1,2}, M. AZUMA¹

¹Department of Microbiology and ²Department of Pediatrics, [Asahikawa Medical College] 4-5 Nishikagura, Asahikawa 078-8510, Japan

Received October 6, 1998; accepted December 8, 1998

Summary. — To analyze the difference in the degree of divergence between genes from identical herpesvirus species, we examined the nucleotide sequence of genes from the herpes simplex virus type 1 (HSV-1) strains VR-3 and 17 encoding thymidine kinase (TK), deoxyribonuclease (DNase), protein kinase (PK; UL13) and virion-associated host shutoff (vhs) protein (UL41). The frequency of nucleotide substitutions per 1 kb in TK gene was 2.5 to 4.3 times higher than those in the other three genes. To prove that the polymorphism of HSV-1 TK gene is common characteristic of herpesvirus TK genes, we compared the diversity of TK genes among eight HSV-1, six herpes simplex virus type 2 (HSV-2) and seven varicella-zoster virus (VZV) strains. The average frequency of nucleotide substitutions per 1 kb in the TK gene of HSV-1 strains was 4-fold higher than that in the TK gene of HSV-2 strains. The VZV TK gene was highly conserved and only two nucleotide changes were evident in VZV strains. However, the rate of nonsynonymous substitutions in total nucleotide substitutions was similar among the TK genes of the three viruses. This result indicated that the mutational rates differed, but there were no significant differences in selective pressure. We conclude that HSV-1 TK gene is highly diverged and analysis of variations in the gene is a useful approach for understanding the molecular evolution of HSV-1 in a short period.

Key words: herpes simplex virus; varicella-zoster virus; nucleotide sequence; polymorphism; molecular evolution

Introduction

The DNA of 13 herpesviruses has been completely sequenced and many single genes of herpesviruses have been extensively analyzed over the past two decades (McGeoch and Cook, 1994; McGeoch *et al.*, 1995; Nicholas, 1996). The results indicate that these viruses share a common evolutionary origin and have extensively diverged. McGeoch and Cook (1994) constructed a robust phylogenetic tree for the alphaherpesviruses (*Alphaherpesvirinae* subfamily)

based on the molecular sequence of glycoprotein B (gB), and inferred a divergence time of 6.6 million years for HSV-1 and HSV-2. The mutation rate of HSV-1 DNA has been estimated based on analyses of the restriction sites of HSV-1 DNAs of several isolates, and viral and human populations are thought to have co-evolved over the last 0.1 million years (Sakaoka *et al.*, 1994).

Nucleotide sequence variation in virus strains has been analyzed using restriction endonuclease cleavage sites. Such variation among HSV genomes has been applied to trace an epidemic of this virus in human populations (Roizman and Tognon, 1982, 1983), which incidentally identified considerable diversity in these genomes (Whitley *et al.*, 1982; Sakaoka *et al.*, 1994, 1995). In contrast, VZV genomes are considerably less diverse (Hondo *et al.*, 1987), suggesting that the evolutionary rates of HSV and VZV differ.

In this study, we sequenced TK and DNase (UL12) genes of three HSV-1 strains, as well as PK (UL13) and vhs protein

*Corresponding author.

Abbreviations: DNase = deoxyribonuclease; gB = glycoprotein B; HSV = herpes simplex virus; ORF = open reading frame; PCR = polymerase chain reaction; PK = protein kinase; TK = thymidine kinase; vhs = virion-associated host shutoff; VZV = varicella-zoster virus

(UL41) genes of the HSV-1 VR-3 strain to define variations among HSV-1 genes and strains. Moreover, we evaluated variations in the nucleotide sequences of the HSV-2 and VZV TK genes, and compared them with that of the HSV-1 TK gene.

Materials and Methods

Cells and viruses. HSV and VZV were propagated in African green monkey kidney cells (Vero) and human embryonic lung fibroblast (HEL) cells, respectively, which were cultured in Eagle's Minimum Essential Medium containing 10% calf serum and 0.15% sodium bicarbonate.

The VR-3 strain of HSV-1 and the UW268 strain of HSV-2 were supplied by the American Type Culture Collection, Rockville, MD, USA. The WT51, KH169 and TAS strains of HSV-1 were clinically isolated from patients with herpes keratitis (WT51 and KH169 strains) or facial herpetic lesions (TAS strain) and passaged a few times in our laboratory (Machida *et al.*, 1991). The KBY and SKN strains of HSV-2 were clinical isolates from female genital herpetic sites, generously provided by Dr. T. Kawana, Department of Obstetrics and Gynecology, University of Tokyo Branch Hospital, Tokyo, Japan. The HSV-2 SL strain was a clinical isolate obtained from Dr. M.P. Langford, Department of Ophthalmology, Louisiana State University Medical Center-Shreveport, Shreveport, LA, USA (Mahjoub *et al.*, 1989; Langford *et al.*, 1990).

The YS strain of VZV was as described (Sakuma, 1984; Lacey *et al.*, 1991). The A-11, A-21, 4-1-1 and 4-2-1 strains were isolated in our laboratory from patients with herpes zoster, and cells infected with these strains were stored at -80°C after less than 5 passages in HEL cells.

DNA sequencing. Nucleotide sequences of HSV-1 (TK gene of WT51, KH169 and TAS strains; DNase gene of VR-3, WT51 and KH169 strains; PK and vhs genes of VR-3 strain), HSV-2 (TK gene of KBY, SKN and SL strains) and VZV (TK gene of A-11, A-12, 4-1-1 and 4-2-1 strains) genes were determined by polymerase chain reaction (PCR)-directed sequencing method (Carothers *et al.*, 1989; Suzutani *et al.*, 1995). Briefly, a double-stranded template DNA for sequencing reactions was prepared by PCR. The PCR products were purified by agarose gel electrophoresis and used as templates. Nucleotide sequences were determined by cycle sequencing using a commercial kit (Sequencing PRO™; Toyobo Co. Ltd., Osaka, Japan) with [α -³²S]dATP (>37 TBq/mmol, Amersham International plc.) according to the manufacturer's instructions. A series of oligonucleotide primers corresponded to sequences within HSV-1, HSV-2 and VZV TK, and HSV-1 DNase genes (Table 1) (Lacey *et al.*, 1991; Suzutani *et al.*, 1995).

Database. Nucleotide sequence cited from the literature or GenBank (reference, accession number) are as follows: HSV-1 TK gene of the 17 (McGeoch *et al.*, 1988; X14112, D00317, D00374 and S40593), C1101 (Wagner *et al.*, 1981; V00470), SC16 (Graham *et al.*, 1986; X03764), McKnight (McKnight, 1980; V00470) and VR-3 (Suzutani *et al.*, 1995; AB009254) strains; HSV-1 DNase, PK and vhs genes of the 17 strain (McGeoch *et al.*, 1988; X14112, D00317, D00374 and S40593); HSV-2 TK gene of the 333 (Kit *et al.*, 1983; Swain and Galloway, 1983; X01712, V02225), HG52 (Dolan *et al.*, 1998; Z86099) and UW268 (Suzutani *et al.*, 1995;

Table 1. Primers used in PCR and sequencing of the HSV DNase gene

Specificity and name	Position* and direction	Sequence
K-UP1	27057-27037	ACAAGGCGATACTGTCGTGG
K-1'	26773-26749	TAACAGTCTTCCGCTGACAACCAC
K-1	26609-26589	ACGCCTCAGGGCCTCCGACC
K-1.2	26379-26356	GATTATCTGTGTTCCCTGGCCGT
K-2	26309-26289	TATGGCGCCTGACGCGCCGC
K-3	26009-25989	GCGCGGACGACGGCGGCGAG
K-4	25709-25689	CCCCGGAGGCGTTCGGGGCA
K-5	25409-25389	ACCCCGTCACCCGAACCTTT
K-6	25219-25197	GTCCATTCTCCCGAACCAGGCC
K-R1	26709-26729	AGCTGATGTCGTCTGGGGTG
K-R1'	26565-26588	CCAGGAGATAGCGGAATGTCTGG
K-R2	26409-26429	AGGGGCGCGGAGGCGCCATA
K-R3	26109-26129	GTGCTTCAGCCTCTGGGGCG
K-R4	25809-25829	CAGGGGGGTCTTGGGGACTG
K-R5	25509-25529	CGAGACAACGCGCTATTTA
K-R6	25209-25229	GAGAATGGACGCTTGTCTGG
K-R7	24986-25008	ACGACGTCAAAGTCGTGGGCGG

*Position of primer on the HSV-1 genome (McGeoch *et al.*, 1988).

AB009256) strains; VZV TK gene of Dumas (Davison and Scott, 1986; X04370), YS (Lacey *et al.*, 1991; AB009253) and 9883 (Lacey *et al.*, 1991; AB009252) strains.

The nucleotide sequences determined in this paper will appear in DDBJ, EMBL and GenBank databases under the Acc. Nos. listed in Table 2.

Results

Analysis of nucleotide sequence variations in TK, DNase, PK and vhs genes of HSV-1

We analyzed nucleotide sequence variations in the HSV-1 genome using HSV-1 genes encoding TK, DNase, PK and

Table 2. List of the accession numbers for the nucleotide sequences determined in this paper

Virus	Gene	Strain	Accession number
HSV-1	TK	WT51	AB009258
		KH69	AB009259
		TAS	AB009260
	DNase	VR-3	AB009264
		WT51	AB009265
		KH169	AB009266
		VR-3	AB009268
HSV-2	PK	VR-3	AB009267
		VR-3	AB009267
	TK	KBY	AB009261
		SKN	AB009262
		SL	AB009263

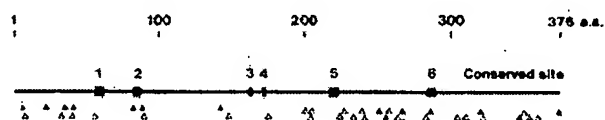


Fig. 1

Positions of nucleotide and amino acid substitutions in HSV-1 TK genes of eight strains and conserved site in herpesvirus TKs. Nucleotide substitutions (empty triangles); amino acid substitutions (full triangles). The HSV-1 strains are described in Table 3.

vhs protein. The reference for this study was the 17 strain, of which the genome has been completely sequenced (McGeoch *et al.*, 1988).

The average numbers of the nucleotide and amino acid substitutions in entire HSV-1 TK gene consisting of 1131 bases and 376 amino acids were 12.9 and 5.4, respectively (Table 3). The positions of nucleotide substitutions and 6 conserved domains in herpesvirus TKs described by Balasubramaniam *et al.* (1990) are summarized in Fig. 1. Variations in amino acid sequence of the TK gene tended to concentrate within 40 codons (codons 224 – 264) located between conserved sites 5 and 6, and within 70 codons at the amino-terminus. Only a switch from asparagine to glutamic acid at codon 286 among a total of 15 amino acid substitutions in the VR-3 TK gene was located in

a conserved domain. The enzymatic characteristic of VR-3 TK and the sensitivity of the VR-3 strain to anti-herpesvirus compounds were the same as those of other HSV-1 strains as described (Suzutani *et al.*, 1988a,b, 1995), indicating that the variation in conserved site 6 did not influence the enzymatic activity.

In the case of DNase gene, nucleotide substitutions were uniformly located throughout the entire gene. The average numbers of nucleotide and amino acid changes in whole gene were 11.8 and 3.0, respectively, representing about half the frequency of those in the TK gene, considering the size differences between the two genes (Table 4, Fig. 3).

We theoretically analyzed these results to understand the basis of the differences in the number of nucleotide and amino acid substitutions between the TK and DNase genes. The TK open reading frame (ORF) consists of 377 codons. We surveyed single nucleotide substitutions in 375 codons of the TK gene, except for the start and stop codons in which mutations change the size of the TK ORF. Three possible nucleotide substitutions for every nucleotide of the 1125 nucleotides in the 375 codons result in 3375 possible mutants possessing a single nucleotide change. These mutations would consist of 2414 nonsynonymous, 867 synonymous and 94 nonsense mutations. In the same manner, 5625 mutations could be caused by a single nucleotide change in HSV-1 DNase gene, and the probability values of amino acid substitutions, silent mutations and changes into stop codons were 4005, 1482 and 138, respectively. The

Table 3. Pairwise comparison of nucleotide and amino acid substitutions in the TK gene between HSV-1 strains

	17	SC16	C1101	*	VR-3	WT51	KH169	TAS	Country	Reference for sequence data
17	–	7	6	7	8	5	5	4	UK	McGeoch <i>et al.</i>
SC16	14	–	5	4	9	6	2	5	UK	Graham <i>et al.</i>
C1101	13	9	–	7	10	3	5	2	USA	Wagner <i>et al.</i>
*	22	16	19	–	7	6	2	5	USA	McKnight
VR-3	19	15	18	19	–	9	7	8	USA	Suzutani <i>et al.</i>
WT51	11	9	4	17	16	–	4	1	Japan	This paper
KH169	13	7	12	17	18	10	–	3	Japan	This paper
TAS	10	8	3	16	15	1	9	–	Japan	This paper

*The strain was not named in the paper (McKnight, 1980).

Table 4. Pairwise comparison of nucleotide and amino acid substitutions in the DNase gene between HSV-1 strains

	17	VR-3	WT51	KH169	Country	References for sequence data
17	–	3	0	3	UK	McGeoch <i>et al.</i>
VR-3	13	–	3	6	USA	This paper
WT51	7	15	–	3	Japan	This paper
KH169	8	16	8	–	Japan	This paper

Figures below and above the diagonal are numbers of nucleotide and amino acid substitutions, respectively.

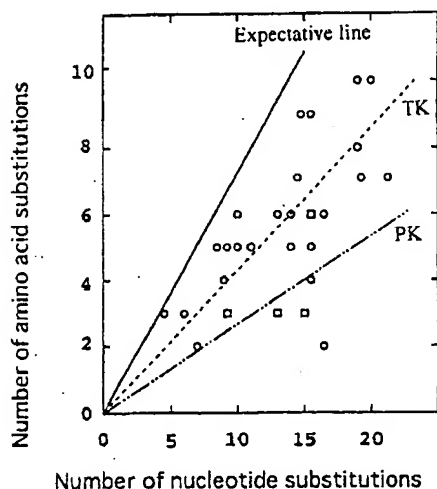


Fig. 2

Experimental and theoretical correlations between numbers of nucleotide and amino acid substitutions

The region between expectative and experimental lines consists of a population which could not survive by natural selection.

experimental and expectative results in terms of correlation between the numbers of nucleotide and amino acid substitutions are shown in Fig. 2. In this figure, the space between the expectative and experimental lines may consist of a population that could not survive because the changes were lethal for the viruses. Our results suggest that the selective pressure was more severe against the HSV-1 DNase than the HSV-1 TK gene ($P < 0.001$).

In order to clarify whether the frequency of mutation in the TK gene is higher than those in other HSV-1 genes that encode enzymes, we analyzed the PK (UL13) and vhs (UL41) genes of the VR-3 strain. Eleven nucleotides in the PK gene and 6 nucleotides in the vhs gene were substituted. The average values of the frequency of nucleotide changes were 7.1 and 4.1 per 1 kb for PK and vhs genes, respectively, and were 42% and 24% of that of TK gene (Fig. 3).

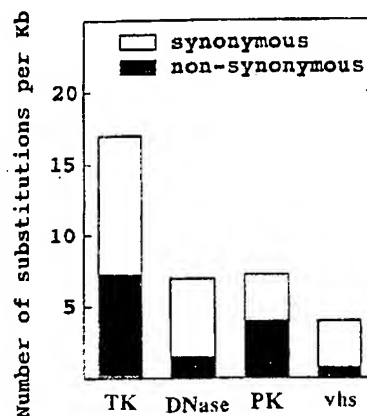


Fig. 3

Numbers of nucleotide and amino acid substitutions in TK, DNase, PK and vhs protein genes of HSV-1 per 1kb

Results obtained by comparing sequences from the VR-3 and 17 strains (McGeoch *et al.*, 1988; Suzutani *et al.*, 1995).

Analysis of variations in HSV-2 TK and VZV TK genes

The above results showed that many variations have accumulated in the TK gene, indicating that this gene is a suitable target for molecular evolutionary analysis of herpesviruses in a short period. Therefore, we analyzed HSV-2 TK and VZV TK genes to compare the diversities of three human alphaherpesviruses. The average numbers of nucleotide and amino acid substitutions in the whole HSV-2 TK gene were 3.2 and 1.6, respectively, which were 25% and 30% of those of the HSV-1 TK gene (Table 5). However, nucleotide sequences of 5 clinically isolated VZVs from Japanese patients were identical and had just two nucleotide changes compared to the Dumas strain, which were silent mutations (T:A to C:G at nt 64989) and a C:G to T:A at nt 65669 causing leucine to be replaced by serine at aa 288 (Davison and Scott, 1986). These results suggested that the

Table 5. Pairwise comparison of nucleotide and amino acid substitutions in the TK gene between HSV-2 strains

	333	UW68	HG52	KBY	SKN	SL	Country	References for sequence data
333	—	0	1	2	1	1	USA	Kit, Swain <i>et al.</i>
UW68	2	—	1	2	1	1	USA	Suzutani <i>et al.</i>
HG52	2	4	—	3	0	2	UK	Dolan <i>et al.</i>
KBY	2	4	4	—	3	3	Japan	This paper
SKN	2	4	2	4	—	2	Japan	This paper
SL	2	4	4	1	4	—	USA	This paper

Figures below and above the diagonal are numbers of nucleotide and amino acid substitutions, respectively.

highly variable nucleotide sequence in the TK gene is characteristic of HSV.

The ratios of amino acid changes to nucleotide changes in the TK gene of HSV-1 and HSV-2 were 41.8% and 47.9%, respectively, indicating that the intensities of selective pressure against these TKs were essentially equal.

Discussion

The molecular evolution of herpesviruses has been analyzed by comparing closely related genes within species (McGeoch and Cook, 1994; McGeoch *et al.*, 1995). In these studies, the nucleotide sequence determined from one strain was cited as representative of species, although many variations were predicted from polymorphism of restriction endonuclease cleavage sites, especially in HSV-1 (Roizman and Tognon, 1982; Whitley *et al.*, 1982; Sakaoka *et al.*, 1994). In the present study, we analyzed variations in the DNA sequences of four enzyme genes of several strains to understand molecular evolution within a species and biological differences among HSV-1, HSV-2 and VZV.

The nucleotide and amino acid sequences of the TK gene were more diverse than those of the PK, DNase and vhs genes. This finding indicates that the mutation rates varied among HSV-1 genes and that the many substituted nucleotides in HSV-1 TK do not effect viral survival. The four enzymes evaluated in this study are not essential for virus replication *in vitro*, although the yield of DNase-deficient virus was $10^2 - 10^3$ times lower than that of the wild type (Field and Wildy, 1978; Kwong *et al.*, 1988; Weller *et al.*, 1990; Coulter *et al.*, 1993; Shao *et al.*, 1993; Overton *et al.*, 1994; Strelow and Leib, 1995; Bronstein and Weber, 1996; Martinez *et al.*, 1996). However, these enzymes might be essential for replication *in vivo* and for survival under selective pressures (Jamieson *et al.*, 1974; Meignier *et al.*, 1988; Nishiyama *et al.*, 1992). Therefore, the mutation rates of these enzyme genes in a single species may reflect differences in the structural stringency required for individual enzyme activities and the location of these enzymes in infected cells and/or virion. Thus many natural variations in the amino acid sequence of active TK should exist and indeed, the polymorphism has been indicated to some degree by isolating several active enzymes from an HSV-1 TK population by random sequence mutagenesis (Munir *et al.*, 1992, 1993). Based on this phenomenon, the HSV-1 TK gene may be useful as a sensitive target for analyzing the molecular evolution of HSV-1 in a short period.

We examined biological and evolutionary differences among HSV-1, HSV-2 and VZV by comparing the diversity of TK genes among viral species. The frequency of nucleotide substitutions per 1 kb in the TK gene of

HSV-1 strains was 4 times higher than that in HSV-2 strains, and nucleotide changes in VZV strains were rare. These results were obtained by analyzing a limited number of isolates from mainly Japanese patients. However, our results may well reflect the nature of variation in the sequence of DNA of HSV-1, -2 and VZV, because the numbers of nucleotide changes among isolates from Japanese patients were comparable with those among Japanese and American (SL and C1101) or British (Dumas and 9883) strains.

Differences in the frequency of nucleotide changes in herpesvirus DNA could be caused by the following.

(i) Fidelity of DNA replication: Most nucleotide substitutions occur during DNA replication (Alberts *et al.*, 1995). Therefore, mutation rates among a viral species would depend on the fidelity of DNA polymerase and viral replication frequency during primary and recurrent infections in one generation of humans. It has not been estimated and may be impossible to determine how many times a latent virus is reactivated in humans, because the reactivation does not always cause clinical disease (Bolognese *et al.*, 1976; Frenkel *et al.*, 1978; Kamayama *et al.*, 1989). Moreover, it is impossible to estimate how many rounds of replication occur following a single reactivation event *in vivo*. Accordingly, the absolute number of virus replication cycles and reactivations cannot be addressed, but our results suggest that HSV-1 is reactivated and replicates more frequently than HSV-2 and that subclinical reactivation of VZV is rare.

(ii) Epidemic behavior: HSV infection appears to spread through close contact with contaminated secretions, although the source of the virus in most infections cannot be identified (Rawls, 1985). Therefore, one strain of HSV seems to be maintained in a small group of humans. On the other hand, VZV infection tends to be epidemic. The epidemic behavior of HSV and VZV might be one factor affecting the frequency of nucleotide substitutions, because mass infection with VZV can occur in fewer replication cycles than that with HSV.

In this study, we showed that analyzing variations in the sequence of TK genes of clinical HSV isolates is a useful approach to understanding the molecular evolution and biology of this virus.

Acknowledgements. We thank Dr. T. Kawana of the University of Tokyo Branch Hospital, Tokyo, Japan and Dr. M.P. Langford of Louisiana State University Medical Center-Shreveport, Shreveport, LA, USA, for donating HSV isolates.

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